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Epidemiology of equine herpesviruses (EHVs) in Ethiopian equids and invasion characteristics of EHV-1 and EHV-3 in respiratory and genital mucosae

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LIST OF ABBREVIATIONS

BM	Basement membrane
CD	Cluster of differentiation
CFT	Complement-fixation tests
CNS	Central Nervous System
CPE	Cytopathic effect
DC	Dendritic cells
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
E	Early
ECE	Equine coital exanthema
EHM	Equine herpesvirus myeloencephalopathy
EHV-1, -2, - 3, -4, -5	Equine herpesvirus-1, -2, -3, -4, -5
EMPF	Equine multinodular pulmonary fibrosis
FITC	Fluorescein isothiocyanate
g	Glycoprotein
h	hour
IE	Immediate early
IF	Immunofluorescence
Ig	Immunoglobulin
L	Late
LC	Langerhans cells
ORF	Open reading frames
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pi	Post inoculation
RK13	Rabbit kidney cells

ROCK	Rho-associated coiled coil kinase
RPMI	Roswell Park Memorial Institute
RSD	Arginine-Serine-Aspartic acid
SD	Standard deviation
SNP	Single Nucleotide Polymorphism
SNT	Serum-neutralizing test
SPSS	Statistical package for the social sciences
$TCID_{50}$	Tissue culture infectious dose with a 50% endpoint
TUNEL	Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling
VNT	Virus neutralization test

CHAPTER 1

INTRODUCTION

1.1. Research background

Working equids (horses, mules, and donkeys) have a great significance in the development of Ethiopia, where they have an essential role in reducing poverty, providing food security, and enhancing rural development. These equids perform numerous activities on a daily basis, including the transportation of goods, people, and construction materials, as well as being used in agricultural and tourism activities. The United Nation Food and Agriculture Organization estimates that Ethiopia has more than 6 million donkeys, the second largest donkey population in the world next to China with 1.9 million horses and over 350, 000 mules (Anonymous, 2010). Infectious diseases compromise the health and welfare of working equids, which in turn threatens the livelihoods of the most vulnerable members of the society (Stringer et al., 2015). However, there are limited or no data quantifying the occurrence, prevalence, and distribution of many infectious diseases in working equids in developing countries. In Ethiopia, previous studies documented that the major factors that affect the health status and performance of working equids includes harness related wounds and sores (Biffa and Woldemeskel, 2006), coughing and nasal discharge (Laing et al., 2016), epizootic lymphangitis (Ameni, 2006; Scantlebury et al., 2013), African horse sickness (Ayelet et al., 2013; Aklilu et al., 2014), strangles (Laing et al., 2016), helminthiasis (Seyoum et al., 2015; Sheferaw and Alemu, 2013), and ocular diseases (Scantlebury et al., 2013). These diseases are widely distributed in different parts of Ethiopia and cause considerable morbidity and mortality. Until recently, the occurrence and distribution of herpesvirus infections in Ethiopian equids had not been known. Since 2009, widespread outbreaks, that are suspected of being associated with EHV-1 have been reported. Currently, the equine herpesviral infections remain an important cause of serious morbidity and mortality in Ethiopian equids.

1.2. Equine herpesviruses

1.2.1. Historical perspective

Equine herpesvirus-1 (EHV-1) was first described by Dimock and Edwards at the Kentucky Agricultural Experimental Station, Lexington in the early 1930s after necropsy examination of an aborted fetus (Dimock and Edwards, 1933). Major antigenic differences between EHV-1 strains have been reported as early as 1959, but were not fully differentiated (Studdert et al.,

1981). Until 1981, two subtypes of this virus were described; EHV-1 subtype 1 was mainly associated with abortion and nervous system disorders and EHV-1 subtype 2 was mainly responsible for respiratory disease. Restriction endonuclease fingerprinting subsequently demonstrated that these two subtypes were closely related, but genetically distinct viruses and therefore, they were reclassified as EHV-1 (formerly subtype 1) and EHV-4 (formerly subtype 2) (Studdert et al., 1981; O'Callaghan and Osterrieder, 2008). EHV-3, the cause of equine coital exanthema (ECE), was first described in Ireland in the early 1900s (Craig and Kehoe, 1921). It was first isolated independently in 1968 in the United States, Canada, and Australia (Bryans, 1968; Girard et al., 1968; Pascoe et al., 1968) and has a worldwide distribution. EHV-2 was first isolated from a horse with upper respiratory tract disease in 1963 (Plummer and Waterson, 1963; Plummer et al., 1969). In 1987, EHV-5 was recognized for the first time from a large viral collection using restriction endonuclease analysis (Browning and Studdert, 1987a).

1.2.2. Taxonomy

Following the recommendations of the *Herpesviridae* study group, the International Committee on Taxonomy of Viruses (ICTV) has summarized herpesviruses within a new order, *Herpesvirales*, which is categorized into three families: the *Herpesviridae*, the *Alloherpesviridae*, and the *Malacoherpesviridae* (Davison, 2010; King et al., 2012). The order has more than 100 members with a wide spectrum of hosts, ranging from humans and other mammals to birds, amphibians, reptiles, and invertebrates (Lacoste et al., 2010). The *Herpesviridae* family contains mammal, reptile, and avian viruses; the *Alloherpesviridae* family contains fish and amphibian viruses and the *Malacoherpesviridae* family contains only the invertebrate bivalve mollusk virus (Davison et al., 2009). The *Herpesviridae* family has a broad range of pathogens that have been grouped into the subfamilies *Alpha-*, *Beta-*, and *Gammaherpesvirinae* based on host range, duration of the reproductive cycle, cytopathology, and characteristic of latent infection (Roizman and Baines, 1991).

The subfamily *Alphaherpesvirinae* has a variable host range, relatively short reproductive cycle, rapid growth in culture, efficient destruction of infected cells, and capacity to establish latent infections (Roizman and Baines, 1991). This subfamily has five genera*: Iltovirus*, *Mardivirus*, *Scutavirus*, *Simplexvirus*, and *Varicellovirus* (King et al., 2012). The EHV-1, -3,

and -4 are members of the *Alphaherpesvirinae* subfamily and belong, along with bovine herpesvirus-1, suid herpesvirus-1, canine herpesvirus-1, felid herpesvirus-1, and Marek's disease virus, and the prototypic virus of the human pathogen varicella-zoster virus, to the genus *Varicellovirus* (King et al., 2012; Davison, 2014). Other equine viruses belonging to this genus are EHV-6 (asinine herpesvirus 1), EHV-8 (asinine herpesvirus 3) and EHV-9 (gazelle herpesvirus) (Table 1).

In contrast, the subfamily *Betaherpesvirinae* has a restricted host range, long reproductive cycle, and the infection progresses slowly in culture. The infected cells frequently become enlarged (cytomegaly) (Roizman and Baines, 1991). Four genera are present within the subfamily *Betaherpesvirinae*: *Cytomegalovirus, Muromegalovirus, Proboscivirus,* and *Roseolovirus* (Davison et al., 2009; King et al., 2012). Initially, EHV-2 and EHV-5 were classified in the *Betaherpesvirinae* subfamily (Roizman and Baines, 1991). However, Telford et al. (1993) demonstrated that the nucleotide sequence of 39 kbp of EHV-2 and 42 kbp of EHV-5 genome shared greater degree of homology with the gammaherpesviruses Epstein-Barr virus and herpesvirus saimiri than with any other herpesviruses, and therefore, they were reclassified in the *Gammaherpesvirinae* subfamily.

The subfamily *Gammaherpesvirinae* comprises viruses that are host-specific *in vitro*, and have a slow growth rate and a high degree of cell-association. This subfamily encompasses the genus *Lymphocryptovirus, Macavirus, Percavirus,* and *Rhadinovirus.* Initially, EHV-2 and EHV-5 were classified in the *Rhadinovirus* sub-family of the *Gammaherpesvirinae*. However, more recently, they were reclassified in the genus *Percavirus* (Ackermann, 2006; Davison, 2002; Davison et al., 2009; King et al., 2012).

Table 1: The known herpesviruses in equids.

Taxon name	Common name	Sub-family	Host	Clinical signs
Equid herpesvirus 1	Equine abortion virus	α	Horse	Respiratory disease, abortion, myeloencephalopathy, chorioretinopathy
Equid herpesvirus 2	Equine herpesvirus 2	γ	Horse	Respiratory disease, conjunctivitis
Equid herpesvirus 3	Equine coital exanthema virus	α	Horse	Coital exanthema
Equid herpesvirus 4	Equine rhinopneumonitis virus	α	Horse	Respiratory disease
Equid herpesvirus 5	Equine herpesvirus 5	γ	Horse	Respiratory disease, multinodular pulmonary fibrosis
Equid herpesvirus 6	Asinine herpesvirus 1	α	Donkey	Lesions on the external genitalia and udder
Equid herpesvirus 7	Asinine herpesvirus 2	γ	Donkey	Not known
Equid herpesvirus 8	Asinine herpesvirus 3	α	Donkey	Respiratory disease
Equid herpesvirus 9	Gazelle herpesvirus	α	Gazelle	Respiratory disease, encephalitis
Asinine herpesvirus 4	Asinine herpesvirus 4	γ	Donkey	Pneumonia
Asinine herpesvirus 5	Asinine herpesvirus 5	γ	Donkey	Pneumonia
Asinine herpesvirus 6	Asinine herpesvirus 6	γ	Donkey	Pneumonia
Zebra herpesvirus	Zebra herpesvirus	γ	Zebra	Pneumonia
Wild ass herpesvirus	Wild ass herpesvirus	γ	Wild ass	Pneumonia

The known herpesviruses appear to share the following significant biological properties (Roizman and Baines, 1991): (1) all herpesviruses specify a large array of enzymes involved in the processing of proteins and in nucleic acid metabolism. Although all herpesviruses specify a DNA polymerase and other proteins involved in DNA synthesis, the exact array of enzymes may vary from one herpesvirus to another; (2) the synthesis of viral DNAs and assembly of capsids occur in the nucleus, acquire an envelope by budding through the nuclear membrane;

(3) production of infectious progeny virus is invariably accompanied by the irreversible destruction of the infected cell; (4) latency is established in their natural hosts.

1.2.3. General structure

The architecture of the member of the family *Herpesviridae* differs significantly with respect to the properties of their DNAs, gene content, and linear arrangement of the genes in viral genomes (Roizman and Baines, 1991). A typical herpesvirion consists of a core containing a linear double stranded DNA, icosahedral capsid surrounded by a structured tegument, and an envelope containing viral glycoprotein spikes on its surface (Figure 1).

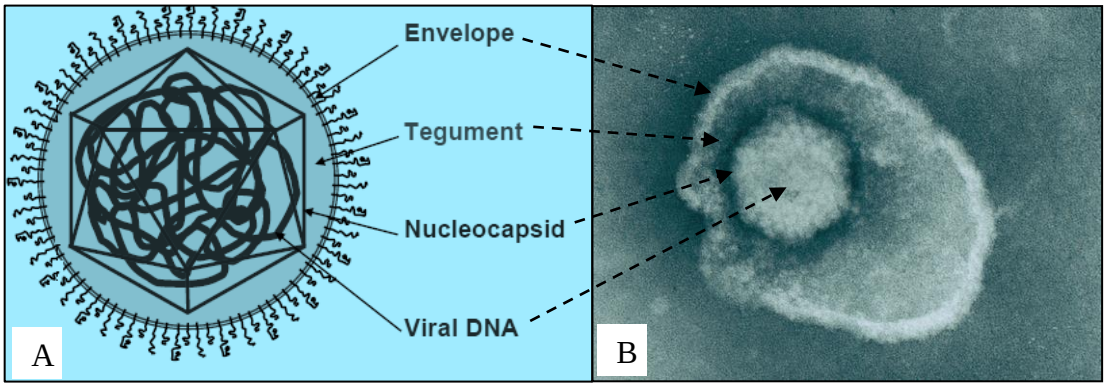

Figure 1: Schematic representation (A) and electron photomicrograph (B) of the morphology of an equine herpesvirus (adapted from Paillot et al., 2008).

Viral genome: EHV-1, -3, and -4 possess linear double-stranded DNA genomes and share the same overall genome conformation with a unique long region (U_L) linked to a unique short region (U_S) that is flanked by an identical pair of inverted repeat regions, the terminal repeat (T_R) and internal repeat (I_R) regions (Telford et al., 1992, 1998; O'Callaghan and Osterrieder, 2008). The EHV-1, -3, and -4 genomes are 150 kbp, 151 kbp, and 146 kbp in length, respectively. All encode 76 homologous genes, with three duplicated genes in EHV-4 and four duplicated genes in EHV-1 and EHV-3 within the repeat regions (Telford et al., 1992, 1998; Sijmons et al., 2014). EHV-1 and EHV-4 are antigenically very closely related. They exhibit 55 - 84% nucleotide sequence homology and the antibodies can cross-neutralize. EHV-3 is the

most divergent of the equine alphaherpesviruses with an overall nucleotide sequence homology with the other viruses ranging from 62.1 - 64.9% (Sijmons et al., 2014).

EHV-2 and EHV-5 are distinct, but closely related equid herpesviruses, sharing approximately 60% nucleotide sequence homology at the nucleotide and amino acid levels (O'Callaghan and Osterrieder, 2008; Fortier et al., 2010). The genome of EHV-2 is a 184 kbp double-stranded DNA genome and has 79 open reading frame (ORF) encoding 77 distinct proteins (Telford et al., 1995). The unique region of EHV-2 contains an unrelated pair of internal, short inverted repeats at separate locations. In contrast, the EHV-5 genome is 179 kbp in size and lacks both the terminal and the internal sequence repeats (Allen and Murray, 2004) (Figure 2).

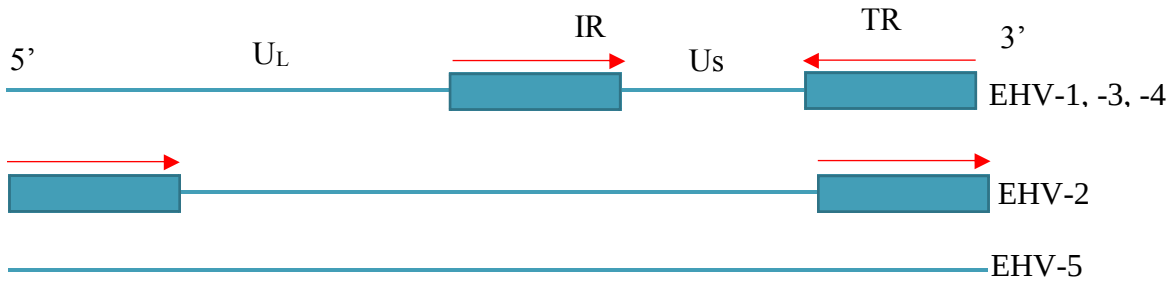

Figure 2: The genome structure of EHVs. Each linear genomic DNA is shown with unique sequences as horizontal lines and repeats as boxes. Orientations of repeats are indicated by arrows. The genomes of EHV-1, -3 and -4 contain two unique regions; the unique long region (U_L) and the unique short region (U_S). U_S is flanked by a repeat region creating identical, but inverted, repeats: the internal (IR) repeat and terminal repeat (TR) regions.

Capsid: In all herpesviruses, the capsid is an icosahedron and has an external diameter of 125 - 130 nm. It consists of 162 capsomeres (12 pentons and 150 hexons) each containing five and six copies of the major capsid protein, respectively (Roizman and Baines, 1991; Brown and Newcomb, 2011).

Tegument: Herpesvirus tegument is a self-supporting structure comprising thousands of densely-packaged protein molecules. It occupies the space between the capsid and the envelope (Owen et al., 2015).

Envelope: The envelope is largely derived from the trans-Golgi network of the host cell and contains viral glycoproteins (Hudnall et al., 2011). For the equid herpesviruses, most of the structural and functional data are available for EHV-1 and EHV-4. The genomes of EHV-1 and EHV-4 encode 12 envelope-anchored surface glycoproteins; namely glycoprotein (g) B, gC, gD, gE, gG, gH, gI, gK, gL, gM, gN, and gp2, which are involved in virus attachment, cell penetration and egress, cell-to-cell spread as well as pathogenicity and virulence (Table 2). Moreover, because of their location in the viral envelope and on the surface of infected cells, glycoproteins are also prime targets of the host's immune response. The gB, gD, gH, gK, and gL are essential glycoproteins which are absolutely required for virus replication in tissue culture (O'Callaghan and Osterrieder, 2008; Osterrieder and Van de Walle, 2010).

Table 2. Equine herpesvirus 1 glycoproteins and their functions

Glyco-proteins	**Function**	**References**
gB	Cell penetration and cell-to-cell spread	Wellington et al., 1996; Neubauer et al., 1997
gC	Mediates cell attachment and egress	Osterrieder, 1999
gD	Cell penetration, cell-to-cell spread, cellular tropism and host cell specificity	Csellner et al., 2000; Sasaki et al., 2011**;** Azab and Osterrieder, 2012
gE	Cell-to-cell spread and virulence	Matsumura et al., 1998; O'Callaghan and Osterrieder, 2008
gG	Viral chemokine binding protein	Bryant et al., 2003; Van De Walle et al., 2007
gH	Integrin-independent cell-to-cell spread	Azab et al., 2012
gI	Cell-to-cell spread and virulence	Matsumura et al., 1998; O'Callaghan and Osterrieder, 2008
gK	Cell-to-cell spread and virus egress	Neubauer and Osterrieder, 2004
gL	Proper folding, intracellular transport, and full functionality of gH	Azab et al., 2012
gM	Cell penetration and cell-to-cell spread	Rudolph and Osterrieder, 2002
gN	gM processing	Osterrieder et al., 1996
gp2	Virus egress and major determinant of virulence	Rudolph and Osterrieder, 2002; O'Callaghan and Osterrieder, 2008

1.2.4. Replication cycle

The alphaherpesvirus replication cycle consists of several steps, including virus entry into the host cell, replication inside the host cell, and release of the virus from the host cell. A schematic representation of the various steps of the alphaherpesvirus replication cycle is shown in Figure 3.

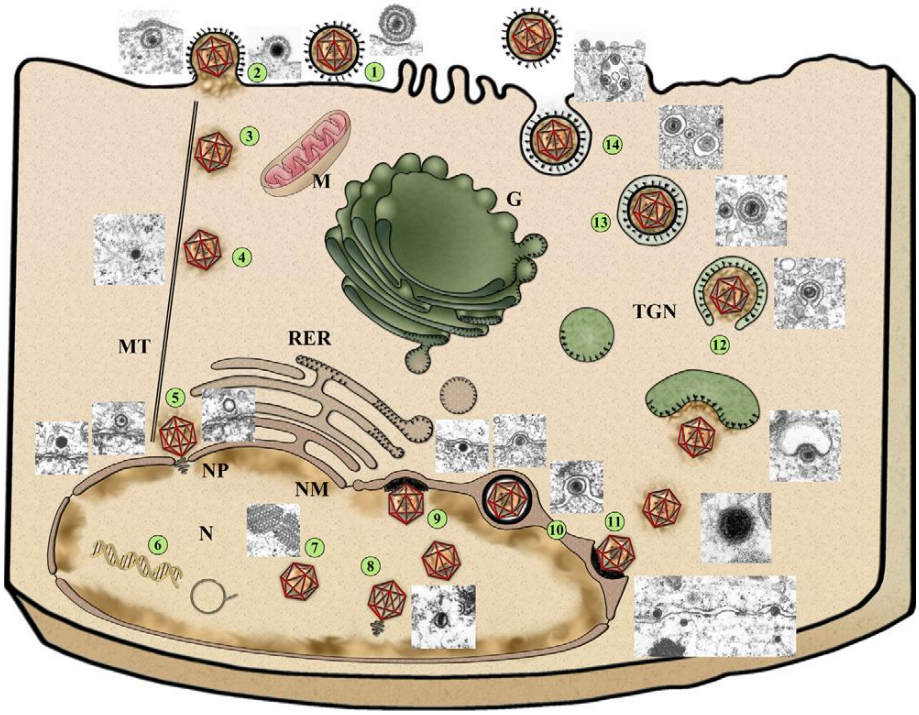

Figure 3: Replication cycle of the alphaherpesviruses (adapted from Mettenleiter et al., 2009). The replication cycle is initiated by the attachment of free virions to the surface of the target cells (1). Then, the viral capsid is released into the cytoplasm (2), transported into the nucleus (N) (3) via the microtubules (MT) (4), and docking at the nuclear pore (NP) (5) where the viral genome is released into the nucleus. In the nucleus, DNA replication and transcription of the viral genes occurs (6). The subsequent packaging of these genomes into newly formed capsids (7) is known as the encapsidation process (8). The nucleocapsids leave the nucleus via budding through the inner nuclear membrane (NM) (9), thereby acquiring their primary envelope, followed by fusion of the envelope of these primary virions located in the perinuclear space (10) with the outer nuclear membrane (11) resulting in the entry of naked nucleocapsids into the cytoplasm. Finally, the nucleocapsids acquire their secondary envelope from the trans-Golgi

network (TGN) (12). After transport to the cell surface (13), vesicle and plasma membrane fuse, releasing a mature, enveloped virion from the cell (14).

Virus attachment and entry

Entry of herpesviruses into the target cells is regulated by interactions with a number of cellular receptors that mediate the attachment, initiate signaling cascades, or trigger virus internalization (Heldwein, 2016). To initiate infection, the virion attaches to cell membranes by the interaction of gC and gB to the heparan sulfate moieties of the cell surface proteoglycans (Shieh et al., 1992; Osterrieder, 1999). The gD is essential for stable binding to cell surface receptors and triggers fusion (Spear and Longnecker, 2003; Van de Walle et al., 2008). After initial attachment, virus entry occurs either by direct fusion of the virus envelope with the plasma membrane in equine dermal cells, endothelial cells, and rabbit kidney cells or via endocytosis in CHO-K1 cells and peripheral blood mononuclear cells (PBMCs) following fusion between the virus envelope and an endosomal membrane (Neubauer et al., 1997; Van de Walle et al., 2008). EHV-1 entry via endocytosis is mediated by cellular αV integrins and arginine-serine-aspartic acid (RSD) motif at amino acid positions 152-154 present in EHV-1 gD (Van de Walle et al., 2008). Recently, Kurtz et al. (2010) and Sasaki et al. (2011) demonstrated that *Equus caballus* major histocompatibility complex class I (MHC-I) can be identified as a cellular entry receptor for the EHV-1. Subsequently, the de-enveloped virus released into the cytoplasm of infected cells, utilizes the microtubule network and the motor protein dynein to move towards the nuclear pore of the cell (Frampton et al., 2010). At the nuclear pores, the nucleocapsid releases its DNA into the nucleus, leaving an empty capsid behind. Successful EHV-1 infection requires the activation of cell signaling molecules, the serine/threonine Rho kinase ROCK1, regardless of the mode of initial entry (Frampton et al., 2007).

Genome replication

Viral DNA replication initiates at approximately 4h post infection and requires the virus-encoded DNA polymerase (O'Callaghan and Osterrieder, 2008). The transcription of viral DNA takes place inside the nucleus, but all viral proteins are synthesized in the cytoplasm. DNA replication occurs with sequential transcription and translation of immediate early (IE), early (E), and late (L) genes. The first set of viral genes to be transcribed are the IE genes, which produce control proteins that stimulate and regulate all the subsequent steps in the replicative

cycle. Their production is essential to stimulate the production of E polypeptides, which are the enzymes and other proteins involved in viral nucleic acid reproduction. Products of IE and E genes transactivate the translation of the L genes, which produce the late proteins, the structural proteins for the virion including the viral capsid and glycoproteins (Klapper and Van Loon, 2010; Slater, 2014).

Virus assembly and egress

The newly synthesized DNA copies have to assemble with the capsid proteins to form nucleocapsids. The host RNA polymerase II transcribes the IE genes; the mRNAs are transported into the cytoplasm where translation occurs. The IE proteins activate transcription of the E genes that are involved in viral DNA replication. Following viral DNA replication, L viral genes start to be transcribed. The L proteins are involved in assembling the capsids within the nucleus (Gruffat et al., 2016). The capsid proteins then translocate to the nucleus of the infected cell. After intranuclear assembly, nucleocapsids bud at the inner leaflet of the nuclear membrane into the perinuclear space, resulting in enveloped particles in the perinuclear space, thus acquiring a primary envelope. The primary envelope then fuses with the outer leaflet of the nuclear membrane thereby releasing naked nucleocapsids into the cytoplasm (Mettenleiter, 2004). The naked nucleocapsids acquire their final tegument in the cytoplasm and tegumented capsids obtain their final envelope by budding into vesicles of the trans-Golgi network (Granzow et al., 2001; Mettenleiter, 2002; Mettenleiter et al., 2006).

1.2.5. Epidemiology

Transmission

Infection with EHV-1, -2, -4, and -5 occurs through the respiratory tract by inhalation of the aerosolized infectious virus, nose-to-nose contact, or contact with fomites (Bell et al., 2006; Hussey and Landolt, 2015), whereas EHV-3 is transmitted through direct skin-to-skin contact during coitus or through secretions containing live virus (Hussey and Landolt, 2015). The presence of EHV-1 in semen of infected stallions has been reported (Hebia-Fellah et al., 2009; Fritsche and Borchers, 2011). Hebia-Fellah et al. (2009) demonstrated EHV-1 DNA in 13% (51/390) of semen samples using conventional PCR test. EHV-1 shedding in semen was also detected on day 20 after the onset of fever in naturally infected stallions using real-time PCR

(Walter et al., 2012). Tearle et al. (1996) recovered EHV-1 from the epididymis and the testis in an experimental study. Although EHV-1 can replicate in the testes and epididymis and be shed in the semen, it is still unclear whether the virus can actually spread by the venereal route.

Host

EHVs circulate in a given horse population via silent or clinical infections or reactivation of latent infections. The horse is the natural host to EHV-1, -2, -3, -4 and -5. Although all breeds of horses are susceptible to EHV-1 infection, clinically EHV-1-affected donkeys and mules have not been reported in previous studies. However, donkeys and mules have shown seroconversion indicating infection with EHV-1 while in contact with affected horses during outbreaks (Pusterla et al., 2012). Although horses and mules are both exposed to EHV-1 during equine herpesvirus myeloencephalopathy (EHM) outbreaks, the clinical neurological signs have only been seen in horses (Pusterla et al., 2012). Mules and donkeys are serve as reservoirs and potential silent shedders of EHV-1 during an outbreak of EHV-1 (Van Maanen, 2002; Pusterla et al., 2012). EHV-1 can occasionally infect non-equid species including llamas (*Lama glama*), alpacas (*Vicugna pacos*), blackbuck (*Antelopa cervicapra*), black bears (*Ursus americanus*), Thomson's gazelles (*Eudorcas thomsoni*), and guinea pigs (*Cavia porcellus*) (Chowdhury et al., 1988; Rebhun et al., 1992; Pagamjav et al., 2007; Wohlsein et al., 2011).

Latency and reactivation

The epidemiological features of EHV infections are: (i) high incidence of respiratory infection early in life with low morbidity, (ii) establishment of lifelong latency in a large proportion of infected equids, and (iii) frequent reactivation of latent virus with subsequent shedding, resulting in transmission to naive hosts (Edington, 1992, Allen et al., 2004; Hussey and Landolt, 2015). EHV-1 establishes latency in the lymphoreticular system, both in circulating leukocytes and in lymph nodes, and in the trigeminal ganglion. EHV-2 is latent in B-lymphocytes, macrophages, and possibly Langerhans cells (Borchers et al., 1997a; Allen and Murray, 2004; Drummer et al., 1996). The sites of EHV-4 latency are sensory ganglia (Borchers et al., 1997b) and lymphoreticular tissues. In contrast, the anatomical sites that harbor latent EHV-3 (Allen and Umphenour, 2004) and EHV-5 (Gilkerson et al., 2015) are unknown. The establishment of latency provides a permanent reservoir for the EHVs which makes total disease eradication highly unlikely.

Periodically, latently infected horses experience reactivation episodes, during which infectious virus is shed into respiratory tract secretions, with the potential of infecting other susceptible horses (Slater, 2014). The physiological factors responsible for the activation of the latent virus are anecdotally associated with stressors including weaning, commingling, transportation, and concurrent infections (Lunn et al., 2009). Recrudescence during times of host stress is thought to result in anterograde axonal transport of virus to the site of initial infection (Pusterla et al., 2012). During latency, the entire viral genome is present within the nucleus of the host cells, but transcription of viral DNA is limited. This renders the virus invisible to immune surveillance (Grinde, 2013).

Prevalence

Estimates of the prevalence of EHV infections strongly vary with viral detection technologies (Lunn et al., 2009). The prevalence of latent EHV-1 infection in horses varies between 54 and 88% (Edington et al., 1994; Allen et al., 2008; Lunn et al., 2009; Pusterla et al., 2010). Latent EHV-2 and EHV-5 infections have been detected in 75 and 88% of foals, respectively (Bell et al., 2006). Information regarding the prevalence of latent EHV-3 is scarce. Barrandeguy et al. (2008) demonstrated reactivation of latent EHV-3 after corticosteroid administration. Surveys to estimate the seroprevalence of EHV infections have revealed the existence of antibodies in most horse populations. The serological surveys of EHV-1 and EHV-4 have always been complicated by the presence of antigenic cross-reactivity and the widespread use of EHV-1 and/or EHV-4 vaccines (Patel and Heldens, 2005). Currently, a type-specific ELISA is available, which can distinguish antibodies to EHV-1 and EHV-4 (Crabb et al., 1995). The seroprevalence of EHV-1 and EHV-4 in horses has been reported between 8 and 85.2% (Gilkerson et al., 1999a; Pusterla et al., 2009) and approximately over 90%, respectively (Aharonson-Raz et al., 2014; Gilkerson et al., 1999b). Information associated with the prevalence of EHV infections in donkeys and mules is limited. EHV-1 has been reported with a seroprevalence of 37.2% (32/86) in mules and 24.2% (31/128) in donkeys using a virus neutralization (VN) test in Turkey (Ataseven et al., 2009).

The genome sequence of EHVs has facilitated molecular epidemiological studies. Sequence analysis of EHV-1 strains revealed a nucleotide variation rate of approximately 0.1% (Nugent et al., 2006; Slater et al., 2006). A region of ORF68 gene of EHV-1 has the highest sequence

variation rate (2%) and has been used as a phylogenetic marker to distinguish an international collection of viruses into six groups (Nugent et al., 2006). This phylogenetic marker helps to trace the source of the EHV-1 disease outbreaks (Barbic et al., 2012), but do not provide any relationship between the phylogenetic groups and the paralytic potential of strains (Slater et al., 2006). The sequence variation of the DNA polymerase gene encoded by ORF30 provides a means of identifying paralytic viruses. EHM is significantly associated with a single nucleotide polymorphism resulting in an amino acid variation of the EHV-1 DNA polymerase (Nugent et al., 2006; Goodman et al., 2007; Van de Walle et al., 2009). EHV-1 encoding aspartic acid (D_{752}) has a higher risk of causing neurological disease than EHV-1 with asparagine (N_{752}) at amino acid position 752 (Nugent et al., 2006). The EHV-1 N_{752} variants have been responsible for approximately 95 to 98% of abortion outbreaks and between 15 and 25% of neurological outbreaks (Lunn et al., 2009). However, recent studies show an increase of the neuropathogenic variant of EHV-1 in abortion outbreaks (Smith et al., 2010; Damiani et al., 2014). The neuropathogenic variants have a longer duration and greater magnitude of viremia and significantly higher number of infected immune cells when compared to non-neuropathogenic variants (Allen and Breathnach, 2006; Vandekerckhove et al., 2010). However, in recent years, an increased number of outbreaks of EHM and a high case fatality rate associated with EHM have been reported (Henninger et al., 2007; Perkins et al., 2009; Goehring et al., 2010; Gryspeerdt et al., 2011). Following a perceived increase in the incidence of EHM outbreaks over the last decade, the US Department of Agriculture classified EHM as a potentially emerging disease of equids (USDA-APHIS, 2007).

1.2.6. Pathogenesis

The equine alphaherpesviruses

Primary EHV-1 and EHV-4 infections occur at the respiratory epithelium, resulting in distinct herpetic lesions of the upper respiratory epithelial mucosa and viral shedding for 10 to 14 days after infection, or even longer in EHM-affected horses (Allen et al., 2004; Lunn et al., 2009). EHV-1 spreads rapidly through the basement membrane of the respiratory mucosal epithelial cells by the misuse of trafficking resident immune cells and hence, the infection becomes systemic. Within 24 to 48 hours after infection, the cell-to-cell spread of EHV-1 results in the presence of virus in respiratory tract lymph nodes where further replication and infection of

leukocytes occurs (Kydd et al., 1994a). T-lymphocytes and monocytic lineage cells are identified as the predominant cell types infected with EHV-1 (Patel et al., 1982; Gryspeerdt et al., 2010; Vandekerckhove et al., 2011; Wilsterman et al., 2011). The cell-associated viremia arises at 24h post infection (Gryspeerdt et al., 2010) and can last till 9 to 23 days. The cell-associated viremia allows the virus to reach the secondary sites of virus replication, particularly the endothelial cells of the pregnant uterus, the central nervous system, and the eye (Edington et al., 1986; Smith et al., 1993, 1996; Lunn et al., 2009; Hussey et al., 2013). This infection of endothelial cells ultimately induces abortion, myeloencephalopathy or chorioretinopathy (Smith et al., 1992; Kydd et al., 1994a, 1994b; Hussey et al., 2013) (Figure 4).

The pathogenesis of EHV-1 abortion involves virus translocation from the circulation into the placenta, which causes infection of endothelial cells of the endometrium, leading to vasculitis, thrombosis, microcotyledonary infarction, and perivascular cuffing (Slater et al., 2006; Smith and Borchers, 2001). Severe infection of the endometrium leads to premature placental separation and abortion of a virologically negative fetus. However, in most cases, the virus can transfer across the uteroplacental barrier and affect the fetal viscera, which in turn leads to expulsion of a fetus with characteristic necrotic lesions and significant virus load (Cullinane et al., 2006).

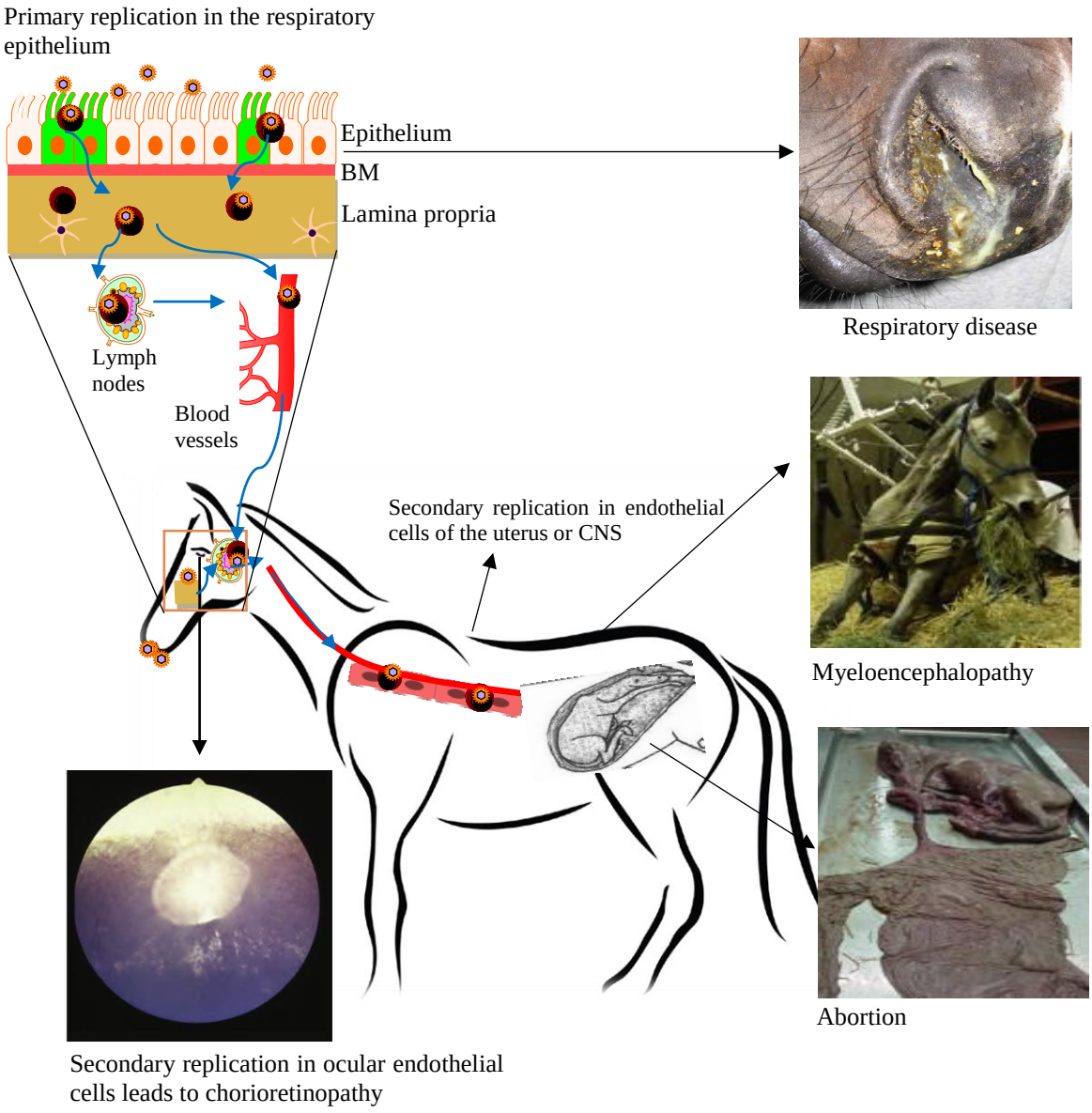

Figure 4: The pathogenesis of EHV-1. The virus enters into the respiratory mucosa by inhalation, causing erosions in the respiratory mucosa. Via a cell-associated viremia, EHV-1 spreads to the underlying tissues and further replicates in the local lymph nodes of the respiratory tract. Hereafter, the virus is transported to the endothelial cells of the pregnant uterus, the central nervous system, or the eye, hence leads to abortion, myeloencephalopathy, or chorioretinopathy.

The leukocyte-associated viremia to endothelial cells of small blood vessels of the brain or spinal cord results in damage to the microvasculature of the central nervous system due to initiation of an inflammatory cascade, vasculitis, microthrombosis, and extravasation of mononuclear cells resulting in perivascular cuffing and local hemorrhage (Edington et al., 1986; Lunn et al., 2009; Pusterla and Hussey, 2014).

In contrast to EHV-1, EHV-4 infection remains in most cases limited to the upper respiratory tract causing a rhinopharyngitis and tracheobronchitis. The differences in the pathogenic potential of the two closely related viruses are mainly associated with (1) the variations in cellular tropism that is mainly determined by gD (absence of RSD motif, which is involved in the infection of PBMCs), (2) the inability of EHV-4 gG to modulate chemokines which are crucial for leukocyte migration, and (3) the rarity of a leukocyte-associated viremia (Osterrieder and Van de Walle, 2010; Vandekerckhove et al., 2011).

EHV-3 is the causal agent of equine coital exanthema (ECE), an acute venereal mucocutaneous disease of horses characterized by the development of papules, vesicles, pustules and ulcers on the vaginal and vestibular mucosa, as well as on the skin of the penis, prepuce, and perineal region. The virus replicates in the stratified epithelium of epidermal tissues present at the mucocutaneous margins and skin. Destruction of the epithelium by the lytic virus infection elicits a vigorous inflammatory response that gives rise to the formation of characteristic cutaneous lesions of ECE (Allen and Umphenour, 2004; Barrandeguy and Thiry, 2012).

The equine gammaherpesviruses

EHV-2 and EHV-5 are most frequently transmitted horizontally to a newborn foal from its dam via the nasopharyngeal route or subsequently, through contact with other foals (Browning and Studdert, 1987; Bell et al., 2006). After inhalation, the virus infects the upper respiratory tract mucosal cells and B-lymphocytes probably at the site of draining lymphoid tissue (Borchers et al., 1997a; Gilkerson et al., 2015). Then, the lymphocyte-associated virus enters the circulation, disseminates systemically, and establishes latency (Allen and Murray, 2004). Periodic reactivation of latent infections can occur throughout the life of the horse, resulting in the spread of infection within a population. However, infections in later life appear mostly to be asymptomatic (Borchers et al., 1997a; Gilkerson et al., 2015).

1.2.7. Clinical signs

Clinical signs associated with EHV-1 and EHV-4

Respiratory disease: Respiratory disease caused by EHV-1 or EHV-4 is typically more widespread among young horses, with disease outbreaks most likely in the period between weaning and 2 - 3 years of age (Gilkerson et al., 2015). Older horses, which are partially immune as a result of earlier infections with EHV-1 or EHV-4, generally show a reduced duration and severity of respiratory tract disease (Wood et al., 2007). Infection is characterized by a short incubation time (< 1 day) followed by transient elevation of body temperature (38.9 - 41.0°C), inappetence, nasal discharge, pharyngitis, depression, enlarged submandibular lymph nodes, and occasional ocular discharge. Affected horses usually give a few coughs, but coughing is not a major feature of uncomplicated equine herpesvirus respiratory disease (Patel and Heldens, 2005; Cullinane et al., 2006; Gilkerson et al., 2015).

Abortion: EHV-1 is recognized as the most common cause of virus-induced abortion in pregnant mares. Pregnant mares infected with EHV-1 usually abort without prior clinical signs, most infection occurs late in gestation (8-11 months) and appear refractory to abortion if the virus is encountered earlier (less than 120 days) in gestation (Lunn et al., 2009; Slater, 2014). Prevalence of EHV-1 abortion in a group of mares varies, depending on management, degree of immunity, virulence of the virus, various stress factors, and the number of exposed susceptible mares in advanced pregnancy (Swerczek and Dennis, 2006).

Myeloencephalopathy: The incubation periods of the EHM ranges from 6 - 8 days (Crabb and Studdert, 1995). The sudden onset and early manifestation of ataxia, paresis and urinary incontinence, the involvement of multiple horses on the premises, and a recent history of fever, abortion or respiratory disease in affected horse populations are typical features of EHM, although there is considerable variation between outbreaks with respect to epidemiological and clinical findings (Wilson, 1997). Commonly, the caudal spinal cord is more severely affected, resulting in weakness of hind limbs, bladder dysfunction, and sensory deficits in the perineal area. The onset of EHM typically occurs, often suddenly, between 6 to 10 days, often suddenly, and severity reaches a peak within 2 to 3 days (Pusterla and Hussey, 2014).

Chorioretinopathy: EHV-1-associated ocular lesions were first described in the late 1980's in llamas and alpacas (Rebhun et al., 1988; House et al., 1991) and then in a mare and foal during natural outbreaks of paralytic EHV-1 and experimental infections (Whitwell and Blunden, 1992; Hussey et al., 2013). EHV-1 infection of the ocular endothelium leads to chorioretinopathy, which causes permanent "shot- gun" lesions in a substantial proportion of infected horses (Hussey et al., 2013). Although the choroidal vasculature is typically hidden by the pigmented retinal epithelium, ocular lesions are not usually visible *in vivo* until one month or later after infection. Therefore, most ocular infections are subclinical and rarely lead to loss of function or even immediate symptoms. Lesions can be focal, multifocal, or rarely diffuse, in which case they affect the entire eye (Hussey et al., 2013; Pusterla and Hussey, 2014; Hussey and Landolt, 2015).

Disease in neonatal foals: Some EHV-1 infected foals are apparently normal at birth but become weak, depressed, and die within 3 days with severe respiratory distress. Other infected foals may survive for a few weeks before succumbing to a variety of secondary bacterial or viral infections. The foals fail to nurse, become lethargic, pyrexic, leukopaenic, hypoxic, and exhibit severe respiratory distress and intractable diarrhea (Allen et al., 2004; Swerczek and Dennis, 2006).

Clinical signs associated with EHV-3

After an incubation period of 5-9 days, lesions appear as small (1-2 mm), raised and reddened papules, which often go unnoticed. Then, typical lesions of coital exanthema progress sequentially to a vesicle, a pustule and, after epidermal sloughing of the necrotic dome of a pustule, a shallow, raw or encrusted erosion or ulcer. The lesions heal within three weeks (Allen and Umphenour, 2004; Barrandeguy and Thiry, 2012). The infection has no effect on fertility and gestation, so the mare will carry the foal to term and deliver a healthy foal (Van der Meulen et al., 2006). Unilateral rhinitis associated with EHV-3 has also been reported (Barrandeguy et al., 2010b). From ECE outbreaks in Argentina, anorectal lymphadenopathy, constipation, tenesmus and evacuation of firm, dry, mucus-covered faces were reported in association with lesions around the anus (Barrandeguy et al., 2010a). Occasionally, stallions with extensive ECE lesions exhibit discomfort, loss of libido and refuse to mount and copulate with mares (Allen and Umphenour, 2004; Barrandeguy and Thiry, 2012).

Clinical signs associated with EHV-2 and EHV-5

The disease association with EHV-2 or EHV-5 infections has been difficult to establish, as these viruses have been identified from clinically normal horses as well as from horses displaying respiratory disease. EHV-2 has been incriminated in mild respiratory disease (Dunowska et al., 2002; Wang et al., 2007; Ataseven et al., 2010; Goehring, 2015), and keratoconjunctivitis (Kershaw et al., 2001; Rushton et al., 2016) while EHV-5 has been linked to occurrence of equine multinodular pulmonary fibrosis (EMPF) (Williams et al., 2007; Dunowska et al., 2014). Cases of EMPF have multiple coalescing nodules of fibrosis in the lung with histologic evidence of interstitial fibrosis and pneumonia with infiltrations of mixed inflammatory cells such as neutrophils and macrophages (Dunowska et al., 2014; Gilkerson et al., 2015).

1.2.8. Diagnosis

Despite a thorough anamnesis and a detailed clinical examination, it is usually not possible to diagnose any of the diseases associated with EHVs with certainty. In this context, a diagnostic laboratory support is necessary. Diagnostic assays used for the diagnosis of EHV infections are virus isolation, immunofluorescence, polymerase chain reaction (PCR)-based assays, and serologic analyses. Virus isolation remains the "gold standard" for laboratory diagnosis of EHV infections and provides unequivocal evidence of the presence of infectious virus in clinical samples such as respiratory tract, blood, fetal and placental tissue samples (Lunn et al., 2009; Slater, 2014; Hussey and Landolt, 2015). EHVs can be isolated on a variety of cell lines such as rabbit kidney cells, equine dermal cells, and equine embryonic lung cells. A positive EHV isolation is characterized by the appearance of cytopathic effects (CPE) in inoculated cultures. EHV-1, -3 and -4-induced CPE are normally detectable within 5 to 7 days of culture (Slater, 2014), whereas CPE of EHV-2 and -5 is usually detected over 3 – 4 passages (Fortier et al., 2010).

PCR-based assays are extremely sensitive and have become key tools in the diagnosis of EHV infections. Positive PCR results can be obtained when virus isolation is negative due to inactivation of the virus or latent stage of the virus. With the use of specific primers, PCR can yield fast and accurate qualitative results. Several conventional PCR tests have been developed for the detection of DNA of the EHVs, with different type-specific primers capable of

distinguishing between the different EHVs (Kirisawa et al., 1993; Léon et al., 2008; Hue et al., 2014). Conventional non-quantitative PCR results can be interpreted as follows (Lunn et al., 2009): (1) a positive EHV-1 test result on a blood sample indicates viremia most probably resulting from an active infection; however, the detection of latent virus cannot be excluded; (2) a negative EHV-1 test result on a blood sample indicates the absence of detectable EHV-1 viremia; (3) a positive EHV-1 test result on a nasal swab sample should be interpreted as indicative of the shedding of infectious virus; (4) a negative EHV-1 test result on a nasal swab indicates the absence of detectable virus shedding. More recently, quantitative real-time PCR-based assays have also been employed for a more sensitive detection and quantitation of viral loads (Lunn et al., 2009; Slater, 2014). Determination of viral load can offer important advantages as it can allow for better characterization of disease stage, assessment of risk of exposure to other horses and monitoring of response to treatment (Lunn et al., 2009).

Immunofluorescence (IF) tests can be used to demonstrate viral antigens in frozen tissue samples rapidly and with high sensitivity and specificity (Slater, 2014). Immunohistochemistry can be performed on paraffin-embedded, formalin fixed tissue sections to demonstrate viral antigens in infected epithelial and endothelial cells and provides valuable confirmation of the cause of vasculitis in the CNS and fetal tissues and placenta (Allen et al., 2004; Slater, 2007).

Cerebrospinal fluid analysis from equids with EHM often reveals xanthochromia and increased protein concentrations, reflecting vasculitis and protein leakage into the cerebrospinal fluid (Van Maanen, 2002). These cerebrospinal fluid changes, in conjunction with characteristic clinical signs, are suggestive, but not diagnostic, of EHM (Slater, 2007).

Serology can also be used to gain a retrospective diagnosis of EHV infections and forms a valuable part of longitudinal surveillance. Serologic testing which demonstrates a 4-fold or greater increase in serum antibody titer, by serum neutralizing (SN) or complement-fixation (CF) tests, on acute and convalescent samples collected 7-21 days apart provides presumptive evidence of an EHV-1 infection (Lunn et al., 2009). CF antibodies are short-lived, usually becoming undetectable by 3 months after infection (Kydd et al., 2006). The CF antibody test is therefore useful as an indicator of recent EHV-1 infection and during outbreaks of disease. In contrast, because of the longevity of SN antibodies (persisting for more than 9 months), SN test

is important for disease prevalence surveys (Kydd et al., 2006). However, neither SN nor CF test can distinguish antibodies to EHV-1 and EHV-4 (Slater, 2014). A commercially available enzyme-linked immunosorbent assay (ELISA), measuring antibodies directed against EHV gG, is capable of differentiating between EHV-1 and EHV-4 infection (Lunn et al., 2009; Slater, 2014).

1.2.9. Prevention and control

Vaccination

Eliminating EHV infections from the equine population is very challenging because of the early establishment of life-long latency, despite the presence of maternal antibody and the ability of the virus to modulate or suppress the host immune system (Kydd et al., 2006). Prevention of infectious diseases relies heavily on the use of vaccination. The purpose of EHV-1 vaccination is (1) to minimize virus replication in the respiratory tract upon infection, thus limiting nasal shedding and the occurrence of respiratory disorders, and (2) to prevent the occurrence of abortion and/or nervous system disorders. Currently, modified live virus and inactivated vaccines are available for protection against EHV-1 and EHV-4-induced disease. These vaccines induce high titers of CF and VN antibody and appear to offer some protection against respiratory disease. They reduce the duration and titer of nasal virus shedding of virus (Goehring et al., 2010; Bresgen et al., 2012). However, they do not reliably block infection, the development of viremia or the establishment of latency. Indeed, EHM has been observed in horses regularly vaccinated against EHV-1 at 3 to 5 monthly intervals (Lunn et al., 2009; Pusterla et al., 2009; Hussey and Landolt, 2015). Currently, extensive research is continuing to develop improved vaccines such as recombinant vaccines and DNA vaccines against EHV-1 and EHV-4. To date, no vaccines are commercially available for preventing EHV-2, EHV-3, and EHV-5 infections.

Management

Rapid implementation of effective management practices in the event of an EHV outbreak is crucially important to prevent the spread of the virus among equids. The strategy for the control of EHV diseases should be a combination of management and hygiene measures supplemented by vaccination. EHV-1 disease control programs have three common goals: (1) prevention of

viral entry into premises; (2) limiting the extent of spread and severity of clinical disease once EHV-1 enters the premises or appears in the herd; and (3) limiting the viral spread to adjacent premises during an outbreak (Allen, 2002; Slater, 2007).

Recommended herd management practices for prevention of abortion or neurological disorders in pregnant mares have been published as a 'code of practice' (Allen et al., 2004). The recommended procedures include (1) segregation of pregnant mares from all other horses on the premises, (2) isolation for a period of not less than 3 weeks of all mares entering the stud farm, including those that are returning after leaving the premises, (3) subdivision of pregnant mares into small physically separated groups for the duration of gestation, and (4) stress reduction by avoiding physiological stress: maintain social structures, avoid prolonged transport, relocation, poor nutrition, parasitism, environmental exposure, and *en masse* weaning of juveniles.

1.3. Mucosa explants to study virus - host interactions

1.3.1. Mucosal explants in research

Experiments in living animals to study host-pathogen interactions is undesirable and raises ethical questions about animal welfare. *In vivo* experiments with large animals are difficult, as purchase and maintenance costs are high and suitable experimental animals are often difficult to obtain. Thus, there is a constant search for *in vitro* models that minimize the number of *in vivo* experiments and the use of experimental animals. The 3R principals (reduction, refinement, replacement) concerning the reduction of the number of experimental animals, the refinement of the experiments to minimize pain and distress, and the replacement of experimental animals by using *ex vivo* models, were set up by Russel and Burch in 1959 (Russell and Burch, 1959). Explant models are powerful *ex vivo* tools permitting controlled experimental manipulation while maintaining microenvironment architecture (Anderson and Jenkinson, 1998). Variation between animals is minimized as tissues can be obtained from the same animal. Explant models may fulfill many of the above-mentioned requirements. In fact, the three-dimensional structure and normal cell-cell contacts are maintained in these models, thereby providing accessible means to mimic the *in vivo* situation. Explant models have proven

to be beneficial for the study of the interaction of bacterial or viral pathogens with the respiratory and/or the genital mucosae of different species such as pigs (Glorieux et al., 2007; Tulinski et al., 2013), horses (Vandekerckhove et al., 2009; Vairo et al., 2013), cows (Steukers et al., 2011), chickens (Reddy et al., 2014), cats (Li et al., 2015), dogs (Li et al., 2016) and humans (Maher et al., 2005; Glorieux et al., 2011; Steukers et al., 2014).

1.3.2. General characteristics of respiratory and genital mucosae

The respiratory epithelium

The respiratory epithelium of the upper respiratory mucosa acts as the first physical barrier that protects against inhaled substances and pathogens. It is composed of the mucosa and the lamina propria. The respiratory epithelium has 5-7 rows of nuclei and a few intraepithelial lymphocytes. The mucosa is lined with pseudostratified ciliated columnar epithelium containing goblet cells, brush cells, basal cells, lymphoreticular tissue aggregates, and myeloid cells (Jeffery and Li, 1997). The lamina propria consists of loose irregular connective tissue with an increased concentration of subepithelial lymphocytes. Ciliated cells possess numerous cilia of uniform thickness. The round nuclei of pyramidal shaped basal cells are oriented in a linear row of the basement membrane and goblet cells located towards the supranuclear zone of the epithelium (Kumar et al., 2000). The mucus overlying the airway epithelium provides further protection of the mucosa by creating a semipermeable barrier that enables the exchange of nutrients, water, and gasses while being impermeable to most pathogens. It allows effective lung clearance through the mucociliary escalator (Vareille et al., 2011). Furthermore, it also contributes to the innate immune defense through antiviral and anti-inflammatory properties and through interaction with other mucus components such as IgA, collectins, and defensins (Vareille et al., 2011).

The vaginal epithelium

The lower reproductive tract represents a unique site for pathogen entry. The vaginal wall of the mare consists of three layers: tunica mucosa-submucosa, tunica muscularis, and tunica adventitia or serosa. The tunica mucosa-submucosa is made up of the non-keratinized stratified squamous epithelium with abundant loose to dense irregular connective tissue below the epithelial lining. Stratified squamous epithelium, consisting of basal, middle, and superficial regions, covers the vaginal mucosa, which is arranged in longitudinal folds (Evans et al., 2009). An abrupt junction exists with the simple columnar epithelium on the protruding cervical folds at the external uterine orifice (McKinnon et al., 2011). The tunica adventitia contains loose connective tissue, blood vessels, nerves and ganglia (McEntee, 1991). The epithelium shows marked variation within the different areas of the vagina; it is thinner in the anterior region than in the vestibule. Epithelial cell proliferation and maturation are under hormonal regulation, and at peak estrogen levels the vaginal epithelium attains its maximum thickness with the superficial cells containing intracytoplasmic glycogen (Patton et al., 2000; Rosenfeld and Heide, 2008). The vaginal mucosa differs from other mucosae with respect to mucus composition, microbiota and innate and adaptive immune mechanisms (Kumamoto and Iwasaki, 2012). The acidic pH, epithelial barrier, mucus and innate immune responses triggered by the commensal flora act in concert to prevent virus infections in the female genital tract against pathogens (Kumamoto and Iwasaki, 2012).

1.3.3. Cell-cell and cell-matrix adhesions

Cells interact with their neighbors and the underlying extracellular matrix via specialized protein complexes that mediate adhesion, maintain structure, and transmit information to the cell interior about the environment (Balda and Matter, 2016). The integrity of the epithelial cell layer(s) that protects multicellular organisms from the external environment is maintained by intercellular junctional complexes composed of tight junctions, anchoring junctions (adherens junctions and desmosomes), and communicating junctions (gap junctions) (Schneeberger and Lynch, 2004).

Tight junctions (zonula occludens) are the most apical of the intercellular junctions and are composed of transmembrane proteins that make contact across the intercellular space and create

a seal to restrict paracellular diffusion of molecules across the epithelial sheet (Sukita et al., 2001; Schneeberger and Lynch, 2004). Tight junctions also have an organizing role in epithelial polarization by limiting the mobility of membrane-bound molecules between the apical and basolateral domains of the plasma membrane of each epithelial cell (Schneeberger and Lynch, 2004; Balda and Matter, 2016).

Anchoring junctions are responsible for the mechanical attachment of the cytoskeleton of a cell to another cell or to the extracellular matrix protein over discrete regions. Functionally, anchoring junctions can be classified into adherens junctions (or zonula adherens) and desmosomes (or macula adherens). Adherens junctions connect bundles of actin filaments from cell to cell to form a continuous adhesion belt, usually just below the tight junctions (Marchiando et al., 2010). Desmosomes connect keratin intermediate filaments from cell-to-cell to form a structural framework of great tensile strength (Green and Simpson, 2007; Marchiando et al., 2010).

Communicating junctions (gap junctions) are highly specialized membrane structures that contain clusters of channels. This organization requires the membranes of two neighboring cells to come close to each other leaving a 2-4 nm gap. The channels provide direct intercellular communication pathways allowing rapid exchange of ions and metabolites (Meşe et al., 2007; Goodenough and Paul, 2009).

1.3.4. Extracellular matrix

The extracellular matrix is the non-cellular component present within all tissues and organs and provides essential physical scaffolding for the cellular constituents and initiates crucial biochemical and biomechanical cues that are required for tissue morphogenesis, differentiation, and homeostasis. Within the mucosa, the extracellular matrix consists of the basement membrane and the lamina propria (Tanzer, 2006; Frantz et al., 2010).

The basement membrane

The basement membranes are specialized extracellular matrices that coat the basal aspect of epithelial and endothelial cells, surround all muscle fibers, fat cells, and peripheral nerve cells. It has a structural role in maintaining tissue architecture and providing anchorage of adjacent cells, functions as a selective barrier to migrating or invading cells and, facilitates filtration or temporary storage of macromolecules (Marinkovich et al., 1993). The basement membrane has three component layers: the lamina lucida, the lamina densa, and the lamina reticularis. Together, the lamina lucida and lamina densa make up the basal lamina. The lamina lucida is a clear area between the epithelium and the lamina densa. The lamina densa is a sheet of connective tissue made up of type IV collagens, laminins, entactin, and heparin sulfate proteoglycans. The lamina reticularis is the interface between the basal lamina and the underlying connective tissue. The predominant collagen is type III, followed by collagens I, V, VI, and VII (Aumailley and Smyth, 1998; Evans et al., 2000; Kruegel and Miosge, 2010; Gartner, 2015).

The lamina propria

The lamina propria of the respiratory mucosa consists of a complex three-dimensional network of collagen, elastin fibers (fibrous proteins) and proteoglycans. This network is located underneath the basement membrane and harbors local cells (fibroblasts), mucus-producing glands, blood and lymphatic vessels and nerve endings (Alberts et al., 2002). The lamina propria of the vagina is well vascularized, but is aglandular (McKinnon et al., 2011).

1.3.5. Mucosal immune cells

Lymphocytes

Intraepithelial lymphocytes associated with the respiratory and genitourinary tract epithelium are the first immune cells to encounter pathogens that have invaded an epithelial surface (Beagley and Husband, 1998). They appear to lie in an intercellular position at all depths of the epithelium, but most numerously near the base of the epithelium (Mair et al., 1987). Within the lamina propria of the respiratory epithelium, scattered small lymphocytes are present, however, their numbers are greatest in areas of the lamina propria adjacent to mucosal lymphoid tissue and just underneath the epithelium (Mair et al., 1987).

Myeloid cells

Granulocytes and monocytes, collectively called myeloid cells, are differentiated descendants from common progenitors derived from hematopoietic stem cells in the bone marrow (Kawamoto and Minato, 2004). They represent the major leukocytes in the peripheral blood. Monocytes maturate into macrophages in various tissues. Macrophages are myeloid immune cells that are strategically positioned throughout the body tissues, where they ingest and degrade dead cells, debris, and foreign material and orchestrate inflammatory processes (Varol et al., 2015).

Monocytes may also differentiate into dendritic cells (DCs) in lymphoid organs and Langerhans cells in the skin, which function as professional antigen-presenting cells (Kawamoto and Minato, 2004). DCs are a heterogeneous population of antigen-presenting cells that initiate and orient immune responses (Steinman and Idoyaga, 2010). They have the ability to acquire antigen in infected tissues, to migrate to secondary lymphoid organs, and to provide the co-receptor signals required for the effective helper and cytotoxic T-cell activation (Carbone and Heath, 2003). A specialized subset of DCs, the Langerhans cells (LCs), are located in the stratified squamous epithelial layer of the skin and within the mucosal epithelial lining of the vagina (Iijima et al., 2007).

Mast cells are highly specialized cells of the myeloid lineage that are commonly encountered in mucosal and epithelial tissues near small blood vessels (Lunn and Horohov, 2004). They play a central role in inflammatory and immediate allergic reactions. They are able to release potent inflammatory mediators such as histamine, proteases, chemotactic factors, and cytokines that act on the vasculature, smooth muscle, connective tissue, mucous glands, and inflammatory cells (Amin, 2012). Mast cells are identified at all levels of the equine respiratory mucosa, with the greatest cell density in the nasopharynx. The majority (up to 94%) of this cell population is located within the connective tissue of the lamina propria and only small numbers are present within the surface epithelium (Mair et al., 1988).

1.4. References

Ackermann, M., 2006. Pathogenesis of gammaherpesvirus infections. Vet. Microbiol. 113, 211–222.

Aharonson-Raz, K., Davidson, I., Porat, Y., Altory, A., Klement, E., Steinman, A., 2014. Seroprevalence and rate of infection of equine influenza virus (H3N8 and H7N7) and equine herpesvirus (1 and 4) in the horse population in Israel. J. Equine Vet. Sci. 34, 828–832.

Aklilu, N., Batten, C., Gelaye, E., Jenberie, S., Ayelet, G., Wilson, A., Belay, A., Asfaw, Y., Oura, C., Maan, S., Bachanek-Bankowska, K., Mertens, P.P.C., 2014. African horse sickness outbreaks caused by multiple virus types in Ethiopia. Transbound. Emerg. Dis. 61, 185–192.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., 2002. Cell junctions, cell adhesion and the extracellular matrix, Molecular biology of the cell. Garl. Sci. New York.

Allen, G., 2002. Epidemic disease caused by Equine herpesvirus 1: recommendations for prevention and control. Equine Vet. Educ. 14, 136–142.

Allen, G., Murray, M., 2004. Equid herpesvirus 2 and equid herpesvirus 5 infections, in: Infectious Diseases of Livestock, (Ed.) JAW Coetzer and RC Tustin. Oxford Press (Cape Town). pp. 860–867.

Allen, G., Umphenour, N., 2004. Equine Coital Exanthema, in: Tustin, J.C. and R. (Ed.), Infectious Diseases of Livestock. Oxford Press, Cape Town, South Africa, pp. 860–867.

Allen, G.P., Bolin, D.C., Bryant, U., Carter, C.N., Giles, R.C., Harrison, L.R., Hong, C.B., Jackson, C.B., Poonacha, K., Wharton, R., Williams, N.M., 2008. Prevalence of latent, neuropathogenic equine herpesvirus-1 in the Thoroughbred broodmare population of central Kentucky. Equine Vet. J. 40, 105–110.

Allen, G.P., Breathnach, C.C., 2006. Quantification by real-time PCR of the magnitude and duration of leucocyte-associated viraemia in horses infected with neuropathogenic vs. non-neuropathogenic strains of EHV- 1. Equine Vet. J. 38, 252–257.

Allen, G.P., Kydd, J.H., Slater, J.D., Smith, K.C., 2004. Equid herpesvirus 1 and equid herpesvirus 4 infections., in: JAW Coetzer and RC Tustin (Ed.), Infectious Diseases of Livestock. Oxford Press, Cape Town, South Africa, pp. 829–859.

Allen, G.P., Murray, M.J., 2004. Equid herpesvirus 2 and equid herpesvirus 5 infections, in:

Coetzer, J., Tustin, and R. (Eds.), Infectious Diseases of Livestocks. pp. 860–867.

Ameni, G., 2006. Epidemiology of equine histoplasmosis (epizootic lymphangitis) in carthorses in Ethiopia. Vet. J. 172, 160–165.

Amin, K., 2012. The role of mast cells in allergic inflammation. Respir. Med. 106, 9–14.

Anderson, G., Jenkinson, E.J., 1998. Use of explant technology in the study of in vitro immune responses. J. Immunol. Methods 216, 155–163.

Anonymous, 2010. FAOSTAT|© FAO Statistics Division. Http://Faostat.Fao.Org/. Accessed April 10, 2016.

Ataseven, V.S., Bilge-Dagalp, S., Oguzoglu, T.C., Karapinar, Z., Guzel, M., Tan, M.T., 2010. Detection and sequence analysis of equine gammaherpesviruses from horses with respiratory tract disease in Turkey. Transbound. Emerg. Dis. 57, 271–276.

Ataseven, V.S., Dağalp, S.B., Güzel, M., Başaran, Z., Tan, M.T., Geraghty, B., 2009. Prevalence of equine herpesvirus-1 and equine herpesvirus-4 infections in equidae species in Turkey as determined by ELISA and multiplex nested PCR. Res. Vet. Sci. 86, 339–344.

Aumailley, M., Smyth, N., 1998. The role of laminins in basement membrane function. J. Anat. 193, 1–21.

Ayelet, G., Derso, S., Jenberie, S., Tigre, W., Aklilu, N., Gelaye, E., Asmare, K., 2013. Outbreak investigation and molecular characterization of African horse sickness virus circulating in selected areas of Ethiopia. Acta Trop. 127, 91–96.

Azab, W., Osterrieder, N., 2012. Glycoproteins D of equine herpesvirus type 1 (EHV-1) and EHV-4 determine cellular tropism independently of integrins. J. Virol. 86, 2031–44.

Azab, W., Zajic, L., Osterrieder, N., 2012. The role of glycoprotein H of equine herpesviruses 1 and 4 (EHV-1 and EHV-4) in cellular host range and integrin binding. Vet. Res. 43, 1–12.

Balda, M.S., Matter, K., 2016. Tight junctions as regulators of tissue remodelling. Curr. Opin. Cell Biol. 42, 94–101.

Barbic, L., Lojkic, I., Stevanovic, V., Bedekovic, T., Staresina, V., Lemo, N., Lojkic, M., Madic, J., 2012. Two outbreaks of neuropathogenic equine herpesvirus type 1 with breed-dependent clinical signs. Vet. Rec. 170, 227–227.

Barrandeguy, M., Perkins, J., Donough, J.M., Vissani, A., Olguin, C., Thiry, E., 2010a. Occurrence of Equine Coital Exanthema in Mares from an Embryo Transfer Center. J. Equine Vet. Sci. 30, 145–149.

Barrandeguy, M., Thiry, E., 2012. Equine coital exanthema and its potential economic implications for the equine industry. Vet. J. 191, 35–40.

Barrandeguy, M., Ulloa, N., Bok, K., Fernández, F., 2010b. Outbreak of rhinitis caused by equine herpesvirus type 3. Vet. Rec. 166, 178–179.

Barrandeguy, M., Vissani, A., Olguin, C., Becerra, L., Miño, S., Pereda, A., Oriol, J., Thiry, E., 2008. Experimental reactivation of equine herpesvirus-3 following corticosteroid treatment. Equine Vet. J. 40, 593–595.

Beagley, K., Husband, A., 1998. Intraepithelial lymphocytes: origins, distribution, and function. Crit Rev Immunol 18, 237–54.

Bell, S.A., Balasuriya, U.B.R., Gardner, I.A., Barry, P.A., Wilson, W.D., Ferraro, G.L., MacLachlan, N.J., 2006. Temporal detection of equine herpesvirus infections of a cohort of mares and their foals. Vet. Microbiol. 116, 249–257.

Biffa, D., Woldemeskel, M., 2006. Causes and Factors Associated With Occurrence of External Injuries in Working Equines in Ethiopia. Intern J Appl Res Vet MEd 4, 1–7.

Borchers, K., Wolfinger, U., Goltz, M., Broll, H., Ludwig, H., 1997a. Distribution and relevance of equine herpesvirus type 2 (EHV-2) infections population . Arch. Virol 142, 917–928.

Borchers, K., Wolfinger, U., Lawrenz, B., Schellenbach, A., Ludwig, H., 1997b. Equine herpesvirus 4 DNA in trigeminal ganglia of naturally infected horses detected by direct in situ PCR. J. Gen. Virol. 78, 1109–1114.

Bresgen, C., Lämmer, M., Wagner, B., Osterrieder, N., Damiani, A.M., 2012. Serological responses and clinical outcome after vaccination of mares and foals with equine herpesvirus type 1 and 4 (EHV-1 and EHV-4) vaccines. Vet. Microbiol. 160, 9–16.

Brown, J., Newcomb, W., 2011. Herpesvirus Capsid Assembly : Insights from Structural Analysis. Curr Opin Virol 1, 142–149.

Browning, G.F., Studdert, M.J., 1987a. Genomic heterogeneity of equine betaherpesviruses. J. Gen. Virol. 68, 1441–1447.

Browning, G.F., Studdert, M.J., 1987b. Epidemiology of Equine Herpesvirus 2 (Equine Cytomegalovirus). J. Clin. Microbiol. 25, 13–16.

Bryans, J., 1968. The herpesviruses in disease of the horse, in: Proceeding of the 14th Annual Convention of the American Association of Equine Practitioners. Philadelphia, USA, p. 119–125.

Bryant, N.A., Davis-Poynter, N., Vanderplasschen, A., Alcami, A., 2003. Glycoprotein G isoforms from some alphaherpesviruses function as broad-spectrum chemokine binding proteins. EMBO J. 22, 833–846.

Carbone, F.R., Heath, W.R., 2003. The role of dendritic cell subsets in immunity to viruses. Curr. Opin. Immunol. 15, 416–420.

Chowdhury, S.I., Ludwig, H., Buhk, H.J., 1988. Molecular biological characterization of equine herpesvirus type 1 (EHV-1) isolates from ruminant hosts. Virus Res. 11, 127–139.

Crabb, B., Studdert, M., 1995. Equine herpesviruses 4 (equine rhinopneumonitis virus) and 1 (equine abortion virus). Adv Virus Res 45, 153–90.

Crabb, B.S., MacPherson, C.M., Reubel, G.H., Browning, G.F., Studdert, M.J., Drummer, H.E., 1995. A type-specific serological test to distinguish antibodies to equine herpesviruses 4 and 1. Arch Virol 40, 245–58.

Craig, J., Kehoe, D., 1921. Horse pox and coital exanthema. J. Comp. Pathol. Ther. 34, 126–129.

Csellner, H., Walker, C., Wellington, J.E., McLure, L.E., Love, D.N., Whalley, J.M., 2000. EHV-1 glycoprotein D (EHV-1 gD) is required for virus entry and cell-cell fusion, and an EHV-1 gD deletion mutant induces a protective immune response in mice. Arch. Virol. 145, 2371–2385.

Cullinane, A.A., Barr, B., Bernard, W., Duncan, J.L., Mulcahy, G., Smith, I.M., Timoney, J.F., 2006. Infectious diseases, in: The Equine Manual. Elsevier Ltd, pp. 1–111.

Damiani, A.M., de Vries, M., Reimers, G., Winkler, S., Osterrieder, N., 2014. A severe equine herpesvirus type 1 (EHV-1) abortion outbreak caused by a neuropathogenic strain at a breeding farm in northern Germany. Vet. Microbiol. 172, 555–562.

Davison, A., 2002. Comments on the phylogenetics and evolution of herpesviruses and other large DNA viruses. Virus Res. 82, 127–132.

Davison, A.J., 2014. Herpesviruses: General Features, in: Reference Module in Biomedical Research. Elsevier Inc., pp. 1–7.

Davison, A.J., 2010. Herpesvirus systematics. Vet. Microbiol. 143, 52–69.

Davison, A.J., Eberle, R., Ehlers, B., Hayward, G.S., McGeoch, D.J., Minson, A.C., Pellett, P.E., Roizman, B., Studdert, M.J., Thiry, E., 2009. The Order Herpesvirales. Arch. Virol. 154, 171–177.

Dimock, W.W., Edwards, P.R., 1933. Is there a filterable virus of abortion in mares? Kentucky

Agric. Exp. Stn. Bull. 333, 297–301.

Drummer, H., Reubel, G., Studdert, M., 1996. Equine gammaherpesvirus 2 (EHV-2) is latent in B lymphocytes. Arch. Virol 141, 495–504.

Dunowska, M., Hardcastle, M.R., Tonkin, F.B., 2014. Identification of the first New Zealand case of equine multinodular pulmonary fibrosis. N. Z. Vet. J. 62, 226–231.

Dunowska, M., Wilks, C.R., Studdert, M.J., Meers, J., 2002. Equine respiratory viruses in foals in New Zealand. N. Z. Vet. J. 50, 140–147.

Edington, N., 1992. Latency of equine herpesviruses, in: Proceedings of the Sixth International Conference on Equine Infectious Diseases. Cambridge. R&W Publications, Newmarket, pp. 195–200.

Edington, N., Bridges, C.G., Patel, J.R., 1986. Endothelial cell infection and thrombosis in paralysis caused by equid herpesvirus-1: Equine stroke. Arch. Virol. 90, 111–124.

Edington, N., Welch, H.M., Griffiths, L., 1994. The prevalence of latent Equid herpesviruses in the tissues of 40 abattoir horses. Equine Vet J. 26, 140–142.

Evans, M.J., Van Winkle, L.S., Fanucchi, M. V., Toskala, E., Luck, E.C., Sannes, P.L., Plopper, C.G., 2000. Three-dimensional organization of the lamina reticularis in the rat tracheal basement membrane zone. Am. J. Respir. Cell Mol. Biol. 22, 393–397.

Evans, T.J., Constantinescu, G.M., Ganjam, V.K., 2009. Anatomy and Physiology of the, in: Current Therapy in Large Animal Theriogenology. Elsevier Inc., pp. 47–64.

Fortier, G., Van Erck, E., Pronost, S., Lekeux, P., Thiry, E., 2010. Equine gammaherpesviruses: Pathogenesis, epidemiology and diagnosis. Vet. J. 186, 148–156.

Frampton, A.R., Stolz, D.B., Uchida, H., Goins, W.F., Cohen, J.B., Glorioso, J.C., 2007. Equine herpesvirus 1 enters cells by two different pathways, and infection requires the activation of the cellular kinase ROCK1. J. Virol. 81, 10879–10889.

Frampton, A.R., Uchida, H., von Einem, J., Goins, W.F., Grandi, P., Cohen, J.B., Osterrieder, N., Glorioso, J.C., 2010. Equine herpesvirus type 1 (EHV-1) utilizes microtubules, dynein, and ROCK1 to productively infect cells. Vet. Microbiol. 141, 12–21.

Frantz, C., Stewart, K.M., Weaver, V.M., 2010. The extracellular matrix at a glance. J. Cell Sci. 123, 4195–4200.

Fritsche, A.K., Borchers, K., 2011. Detection of neuropathogenic strains of Equid Herpesvirus 1 (EHV-1) associated with abortions in Germany. Vet. Microbiol. 147, 176–180.

Gartner, L.P., 2015. Textbook of Histology, Fourth edi. ed. Elsevier Health Sciences.

Gilkerson, J.R., Bailey, K.E., Diaz-Méndez, A., Hartley, C.A., 2015. Update on Viral Diseases of the Equine Respiratory Tract. Vet. Clin. North Am. Equine Pract. 31, 91–104.

Gilkerson, J.R., Whalley, J.M., Drummer, H.E., Studdert, M.J., Love, D.N., 1999a. Epidemiological studies of equine herpesvirus 1 (EHV-1) in Thoroughbred foals: A review of studies conducted in the Hunter Valley of New South Wales between 1995 and 1997. Vet. Microbiol. 68, 15–25.

Gilkerson, J.R., Whalley, J.M., Drummer, H.E., Studdert, M.J., Love, D.N., 1999b. Epidemiology of EHV-1 and EHV-4 in the mare and foal populations on a Hunter Valley stud farm: Are mares the source of EHV-1 for unweaned foals. Vet. Microbiol. 68, 27–34.

Girard, A., Greig, A.S., Mitchell, D., 1968. A Virus Associated with Vulvitis and Balanitis in the Horse A Preliminary Report. Can J Comp Med. 32, 603–604.

Glorieux, S., Bachert, C., Favoreel, H.W., Vandekerckhove, A.P., Steukers, L., Rekecki, A., van den Broeck, W., Goossens, J., Croubels, S., Clayton, R.F., Nauwynck, H.J., 2011. Herpes simplex virus type 1 penetrates the basement membrane in human nasal respiratory mucosa. PLoS One 6, 10–13.

Glorieux, S., Van den Broeck, W., van der Meulen, K.M., Van Reeth, K., Favoreel, H.W., Nauwynck, H.J., 2007. In vitro culture of porcine respiratory nasal mucosa explants for studying the interaction of porcine viruses with the respiratory tract. J. Virol. Methods 142, 105–112.

Goehring, L.S., 2015. γ-Herpesviruses in Horses and Donkeys, in: Robinson's Current Therapy in Equine Medicine. Elsevier Inc., pp. 155–157.

Goehring, L.S., Landolt, G.A., Morley, P.S., 2010. Detection and Management of an Outbreak of Equine Herpesvirus Type 1 Infection and Associated Neurological Disease in a Veterinary Teaching Hospital. J. Vet. Intern. Med. 24, 1176–1183.

Goehring, L.S., Wagner, B., Bigbie, R., Hussey, S.B., Rao, S., Morley, P.S., Lunn, D.P., 2010. Control of EHV-1 viremia and nasal shedding by commercial vaccines. Vaccine 28, 5203–5211.

Goodenough, D.A., Paul, D.L., 2009. Gap junctions. Cold Spring Harb Perspect Biol 1, a002576–a002576.

Goodman, L.B., Loregian, A., Perkins, G.A., Nugent, J., Buckles, E.L., Mercorelli, B., Kydd, J.H., Palù, G., Smith, K.C., Osterrieder, N., Davis-Poynter, N., 2007. A point mutation in a herpesvirus polymerase determines neuropathogenicity. PLoS Pathog. 3, 1583–1592.

Granzow, H., Klupp, B.G., Fuchs, W., Veits, J., Osterrieder, N., Mettenleiter, T.C., 2001. Egress of alphaherpesviruses: comparative ultrastructural study. J. Virol. 75, 3675–3684.

Green, K.J., Simpson, C.L., 2007. Desmosomes: new perspectives on a classic. J. Invest. Dermatol. 127, 2499–515.

Grinde, B., 2013. Herpesviruses: latency and reactivation - viral strategies and host response. J. Oral Microbiol. 5, 1–9.

Gruffat, H., Marchione, R., Manet, E., 2016. Herpesvirus late gene expression: A viral-specific pre-initiation complex is key. Front. Microbiol. 7, 1–15.

Gryspeerdt, A., Vandekerckhove, A., Doorsselaere, J. Van, Nauwynck, H.J., 2011. Description of an unusually large outbreak of nervous system disorders caused by equine herpesvirus 1 (EHV1) in 2009 in Belgium. Vlaams Diergeneeskd. Tijdschr. 80, 147–153.

Gryspeerdt, A.C., Vandekerckhove, A.P., Garré, B., Barbé, F., Van de Walle, G.R., Nauwynck, H.J., 2010. Differences in replication kinetics and cell tropism between neurovirulent and non-neurovirulent EHV1 strains during the acute phase of infection in horses. Vet. Microbiol. 142, 242–253.

Hebia-Fellah, I., Léauté, A., Fiéni, F., Zientara, S., Imbert-Marcille, B.M., Besse, B., Fortier, G., Pronost, S., Miszczak, F., Ferry, B., Thorin, C., Pellerin, J.L., Bruyas, J.F., 2009. Evaluation of the presence of equine viral herpesvirus 1 (EHV-1) and equine viral herpesvirus 4 (EHV-4) DNA in stallion semen using polymerase chain reaction (PCR). Theriogenology 71, 1381–1389.

Heldwein, E.E., 2016. gH/gL supercomplexes at early stages of herpesvirus entry. Curr. Opin. Virol. 18, 1–8.

Henninger, R.W., Reed, S.M., Saville, W.J., Allen, G.P., Hass, G.F., Kohn, C.W., Sofaly, C., 2007. Outbreak of neurologic disease caused by equine herpesvirus-1 at a university equestrian center. J. Vet. Intern. Med. 21, 157–165.

House, J.A., Gregg, D.A., Lubroth, J., Dubovi, E.J., Torres, A., 1991. Experimental equine herpesvirus-1 infection in llamas (Lama glama). J. Vet. diagnostic Investig. 3, 137–143.

Hudnall, S.D., Stanberry, L.R., Griffiths, P.D., 2011. Human Herpesvirus Infections, in: Tropical Infectious Diseases: Principles, Pathogens and Practice. Elsevier Inc., pp. 355–368.

Hue, E.S., Fortier, G.D., Fortier, C.I., Leon, A.M., Richard, E.A., Legrand, L.J., Pronost, S.L., 2014. Detection and quantitation of equid gammaherpesviruses (EHV-2, EHV-5) in nasal

swabs using an accredited standardised quantitative PCR method. J. Virol. Methods 198, 18–25.

Hussey, G.S., Goehring, L.S., Lunn, D.P., Hussey, S.B., Huang, T., Osterrieder, N., Powell, C., Hand, J., Holz, C., Slater, J., 2013. Experimental infection with equine herpesvirus type 1 (EHV-1) induces chorioretinal lesions. Vet. Res. 44, 1–15.

Hussey, G.S., Landolt, G.A., 2015. Equine Alphaherpesviruses, in: Robinson's Current Therapy in Equine Medicine. Elsevier Inc., pp. 158–161.

Iijima, N., Linehan, M.M., Saeland, S., Iwasaki, A., 2007. Vaginal epithelial dendritic cells renew from bone marrow precursors. Proc. Natl. Acad. Sci. U. S. A. 104, 19061–6.

Jeffery, P.K., Li, D., 1997. Airway mucosa: Secretory cells, mucus and mucin genes. Eur. Respir. J. 10, 1655–1662.

Kawamoto, H., Minato, N., 2004. Cells in focus Myeloid cells. Int. J. Biochem. Cell Biol. 36, 1374–1379.

Kershaw, O., Von Oppen, T., Glitz, F., Deegen, E., Ludwig, H., Borchers, K., 2001. Detection of equine herpesvirus type 2 (EHV-2) in horses with keratoconjunctivitis. Virus Res. 80, 93–99.

King, A.M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J.S.D., 2012. Virus taxonomy: classification and nomenclature of viruses: Ninth Report of the International Committee on Taxonomy of Viruses, Journal of Chemical Information and Modeling. Elsevier Academic Press.

Kirisawa, R., Endo, A., Iwai, H., Kawakami, Y., 1993. Detection and identification of equine herpesvirus-1 and-4 by polymerase chain reaction. Vet. Microbiol. 36, 57–67.

Klapper, P.E., Van Loon, A.M., 2010. Herpesviruses, in: Infectious Diseases. pp. 1550–1564.

Kruegel, J., Miosge, N., 2010. Basement membrane components are key players in specialized extracellular matrices. Cell. Mol. Life Sci. 67, 2879–2895.

Kumamoto, Y., Iwasaki, A., 2012. Unique features of antiviral immune system of the vaginal mucosa. Curr. Opin. Immunol. 24, 411–416.

Kumar, P., Timoney, J.F., Southgate, H.H., Sheoran, A.S., 2000. Light and scanning electron microscopic studies of the nasal turbinates of the horse. Anat. Histol. Embryol. 29, 103–9.

Kydd, J.H., Smith, K.C., Hannant, D., Livesay, G.J., Mumford, J.A., 1994a. Distribution of equid herpesvirus-1 (EHV-1) in respiratory tract associated lymphoid tissue: implications

for cellular immunity. Equine Vet. J. 26, 470–473.

Kydd, J.H., Smith, K.C., Hannant, D., Livesay, G.J., Mumford, J.A., 1994b. Distribution of equid herpesvirus-1 (EHV-1) in the respiratory tract of ponies: implications for vaccination strategies. Equine Vet. J. 26, 466–469.

Kydd, J.H., Townsend, H.G.G., Hannant, D., 2006. The equine immune response to equine herpesvirus-1: The virus and its vaccines. Vet. Immunol. Immunopathol. 111, 15–30.

Lacoste, V., Lavergne, A., de Thoisy, B., Pouliquen, J.F., Gessain, A., 2010. Genetic diversity and molecular evolution of human and non-human primate Gammaherpesvirinae. Infect. Genet. Evol. 10, 1–13.

Laing, G., Christley, R., Stringer, A., Radford, A., Aklilu, N., Newton, R., Pinchbeck, G., 2016. Unraveling the causes of respiratory disease in the working equids of Ethiopia: A cross sectional survey. J. Equine Vet. Sci. 39, S98–S107.

Léon, A., Fortier, G., Fortier, C., Freymuth, F., Tapprest, J., Leclercq, R., Pronost, S., 2008. Detection of equine herpesviruses in aborted foetuses by consensus PCR. Vet. Microbiol. 126, 20–29.

Li, Y., Negussie, H., Qiu, Y., Reddy, V.R.A.P., Mateusen, B., Nauwynck, H.J., 2016. Early events of canine herpesvirus 1 infections in canine respiratory and genital mucosae by the use of ex vivo models. Res. Vet. Sci. 105, 205–208.

Li, Y., Van Cleemput, J., Qiu, Y., Reddy, V.R. a. P., Mateusen, B., Nauwynck, H.J., 2015. Ex vivo modeling of feline herpesvirus replication in ocular and respiratory mucosae, the primary targets of infection. Virus Res. 210, 227–231.

Lunn, D.P., Horohov, D.W., 2004. Equine Immunology. In: Mechanisms of Disease and Principles of Treatment, in: Equine Internal Medicine. Elsevier Inc., pp. 1–58.

Lunn, D.P., Horohov, D.W., Osterrieder, K., Pusterla, N., 2009. EHV-1 Consensus Statement. J Vet Intern Med 23, 450–461.

Maher, D., Wu, X., Schacker, T., Horbul, J., Southern, P., 2005. HIV binding, penetration, and primary infection in human cervicovaginal tissue. Proc. Natl. Acad. Sci. U. S. A. 102, 11504–11509.

Mair, T., Batten, E., Stokes, C., Bourne, F., 1987. The histological features of the immune system of the equine respiratory tract. J Comp Pathol 97, 575–86.

Mair, T., Stokes, C., Bourne, F., 1988. Distribution and ultrastructure of mast cells in the equine respiratory tract. Equine Vet J 20, 54–8.

Marchiando, A., Graham, W., Turner, J., 2010. Epithelial Barriers in Homeostasis and Disease. Annu Rev Pathol 5, 119–44.

Marinkovich, M.P., Keene, D.R., Rimberg, C.S., Burgeson, R.E., 1993. Cellular origin of the dermal-epidermal basement membrane. Dev. Dyn. 197, 255–67.

Matsumura, T., Kondo, T., Sugita, S., Damiani, a M., O'Callaghan, D.J., Imagawa, H., 1998. An equine herpesvirus type 1 recombinant with a deletion in the gE and gI genes is avirulent in young horses. Virology 242, 68–79.

McEntee, M., 1991. Reproductive Pathology of Domestic Mammals. Elsevier, pp. 191–223.

McKinnon, A.O., Squires, E.L., Vaala, W.E., Varner, D.D., 2011. Equine reproduction, 2nd ed. Wiley-Blackwell.

Meşe, G., Richard, G., White, T.W., 2007. Gap junctions: basic structure and function. J. Invest. Dermatol. 127, 2516–2524.

Mettenleiter, T.C., 2004. Budding events in herpesvirus morphogenesis. Virus Res. 106, 167–180.

Mettenleiter, T.C., 2002. Herpesvirus Assembly and Egress. J. Virol. 76, 1537–1547.

Mettenleiter, T.C., Klupp, B.G., Granzow, H., 2009. Herpesvirus assembly: An update. Virus Res. 143, 222–234.

Mettenleiter, T.C., Klupp, B.G., Granzow, H., 2006. Herpesvirus assembly: a tale of two membranes. Curr. Opin. Microbiol. 9, 423–429.

Neubauer, A., Braun, B., Brandmuller, C., Kaaden, O.R., Osterrieder, N., 1997. Analysis of the contributions of the equine herpesvirus 1 glycoprotein gB homolog to virus entry and direct cell-to-cell spread. Virology 227, 281–94.

Neubauer, A., Osterrieder, N., 2004. Equine herpesvirus type 1 (EHV-1) glycoprotein K is required for efficient cell-to-cell spread and virus egress. Virology 329, 18–32.

Nugent, J., Birch-Machin, I., Smith, K.C., Mumford, J.A., Swann, Z., Newton, J.R., Bowden, R.J., Allen, G.P., Davis-Poynter, N., 2006. Analysis of equid herpesvirus 1 strain variation reveals a point mutation of the DNA polymerase strongly associated with neuropathogenic versus nonneuropathogenic disease outbreaks. J. Virol. 80, 4047–4060.

O'Callaghan, D.J., Osterrieder, N., 2008. Herpesviruses of Horses, in: Encyclopedia of Virology (Third Edition). pp. 411–420.

Osterrieder, N., 1999. Construction and characterization of an equine herpesvirus 1 glycoprotein C negative mutant. Virus Res. 59, 165–177.

Osterrieder, N., Neubauer, A., Brandmuller, C., Braun, B., Kaaden, O.R., Baines, J.D., 1996. The equine herpesvirus 1 glycoprotein gp21/22a, the herpes simplex virus type 1 gM homolog, is involved in virus penetration and cell-to-cell spread of virions. J. Virol. 70, 4110–4115.

Osterrieder, N., Van de Walle, G.R., 2010. Pathogenic potential of equine alphaherpesviruses: The importance of the mononuclear cell compartment in disease outcome. Vet. Microbiol. 143, 21–28.

Owen, D.J., Crump, C.M., Graham, S.C., 2015. Tegument assembly and secondary envelopment of alphaherpesviruses. Viruses 7, 5084–5114.

Pagamjav, O., Yamada, S., Ibrahim, E.-S.M., Crandell, R. a, Matsumura, T., Yamaguchi, T., Fukushi, H., 2007. Molecular characterization of equine herpesvirus 1 (EHV-1) isolated from cattle indicating no specific mutations associated with the interspecies transmission. Microbiol. Immunol. 51, 313–319.

Paillot, R., Case, R., Ross, J., Newton, R., Nugent, J., 2008. Equine Herpes Virus-1: Virus, Immunity and Vaccines. Open Vet. Sci. J. 2, 68–91.

Pascoe, R., Spradbrow, P., Bagust, T., 1968. Equine coital exanthema. Aust. Vet. J. 44, 485.

Patel, J.R., Edington, N., Mumford, J.A., 1982. Variation in cellular tropism between isolates of Equine herpesvirus-1 in Foals. Arch. Virol. 74, 41–51.

Patel, J.R., Heldens, J., 2005. Equine herpesviruses 1 (EHV-1) and 4 (EHV-4) - Epidemiology, disease and immunoprophylaxis: A brief review. Vet. J. 170, 14–23.

Patton, D.L., Thwin, S.S., Meier, A., Hooton, T.M., Stapleton, A.E., Eschenbach, D.A., 2000. Epithelial cell layer thickness and immune cell populations in the normal human vagina at different stages of the menstrual cycle. Am. J. Obstet. Gynecol. 183, 967–973.

Perkins, G.A., Goodman, L.B., Tsujimura, K., Van de Walle, G.R., Kim, S.G., Dubovi, E.J., Osterrieder, N., 2009. Investigation of the prevalence of neurologic equine herpes virus type 1 (EHV-1) in a 23-year retrospective analysis (1984-2007). Vet. Microbiol. 139, 375–378.

Plummer, G., Bowling, C.P., Goodheart, C.R., 1969. Comparison of Four Horse Herpesviruses. J. Virol. 4, 738–741.

Plummer, G., Waterson, A.P., 1963. Equine herpes viruses. Virology 19, 412–416.

Pusterla, N., David Wilson, W., Madigan, J.E., Ferraro, G.L., 2009. Equine herpesvirus-1 myeloencephalopathy: A review of recent developments. Vet. J. 180, 279–289.

Pusterla, N., Hussey, G.S., 2014. Equine herpesvirus 1 myeloencephalopathy. Vet. Clin. North Am. Equine Pract. 30, 489–506.

Pusterla, N., Mapes, S., Madigan, J.E., MacLachlan, N.J., Ferraro, G.L., Watson, J.L., Spier, S.J., Wilson, W.D., 2009. Prevalence of EHV-1 in adult horses transported over long distances. Vet. Rec. 165, 473–475.

Pusterla, N., Mapes, S., Wademan, C., White, A., Estell, K., Swain, E., 2012. Investigation of the role of mules as silent shedders of EHV-1 during an outbreak of EHV-1 myeloencephalopathy in California. Vet. Rec. 170, 465–465.

Pusterla, N., Mapes, S., Wilson, W.D., 2012. Prevalence of latent alpha-herpesviruses in Thoroughbred racing horses. Vet. J. 193, 579–582.

Pusterla, N., Mapes, S., Wilson, W.D., 2010. Prevalence of equine herpesvirus type 1 in trigeminal ganglia and submandibular lymph nodes of equids examined postmortem. Vet. Rec. 167, 376–378.

Rebhun, W.C., Jenkins, D.H., Riis, R.C., Dill, S.G., Dubovi, E.J., Torres, A., A, 1992. An epizootic of blindness and encephalitis associated with a herpesvirus indistinguishable from equine herpesvirus I in a herd of alpacas and llamas. J. Am. Vet. Med. Assoc.

Rebhun WC, Jenkins, D., Riis, R., Dill, S., Dubovi, E., Torres, A., 1988. An epizootic of blindness and encephalitis associated with a herpesvirus indistinguishable from equine herpesvirus 1 in a herd of alpacas and llamas. J Am Vet Med Assoc. 192, 953–6.

Reddy, V.R.A.P., Steukers, L., Li, Y., Fuchs, W., Vanderplasschen, A., Nauwynck, H.J., 2014. Replication characteristics of infectious laryngotracheitis virus in the respiratory and conjunctival mucosa. Avian Pathol. 43, 450–457.

Roizman, B., Baines, J., 1991. The diversity and unity of herpesviridae. Comp. Immunol. Microbiol. Infect. Dis. 14, 63–79.

Rosenfeld, C., Heide, S., 2008. Overview of female reproductive organs, in: Schatten, H., Constantinescu, G.M. (Eds.), Comparative Reproductive Biology. Wiley-Blackwell, pp. 99–109.

Rudolph, J., Osterrieder, N., 2002. Equine herpesvirus type 1 devoid of gM and gp2 is severely impaired in virus egress but not direct cell-to-cell spread. Virology 293, 356–67.

Rushton, J.O., Kolodziejek, J., Nell, B., Weissenböck, H., Nowotny, N., 2016. Keratoconjunctivitis in a group of Icelandic horses with suspected gammaherpesvirus involvement. Equine Vet. J. 48, 427–9.

Russell, W.M.S., Burch, R.L., 1959. The principles of humane experimental technique. Methuen, London, UK.

Sasaki, M., Hasebe, R., Makino, Y., Suzuki, T., Fukushi, H., Okamoto, M., Matsuda, K., Taniyama, H., Sawa, H., Kimura, T., 2011. Equine major histocompatibility complex class I molecules act as entry receptors that bind to equine herpesvirus-1 glycoprotein D. Genes to Cells 16, 343–357.

Scantlebury, C.E., Aklilu, N., Reed, K., Knottenbelt, D.C., Gebreab, F., Pinchbeck, G.L., 2013. Ocular disease in working horses in Ethiopia: a cross-sectional study. Vet. Rec. 172, 99.

Schneeberger, E.E., Lynch, R.D., 2004. The tight junction: a multifunctional complex. Am. J. Physiol. Cell Physiol. 286, C1213–C1228.

Seyoum, Z., Tesfaye, M., Derso, S., 2015. Prevalence, intensity and risk factors of infestation with major gastrointestinal nematodes in equines in and around Shashemane, Southern Ethiopia. Trop. Anim. Health Prod. 47, 1515–1521.

Sheferaw, D., Alemu, M., 2013. Epidemiological study of gastrointestinal helminths of equines in Damot-Gale district, Wolaita zone, Ethiopia. J. Parasit. Dis. 39, 1–6.

Shieh, M.T., WuDunn, D., Montgomery, R.I., Esko, J.D., Spear, P.G., 1992. Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. J. Cell Biol. 116, 1273–1281.

Sijmons, S., Vissani, A., Tordoya, S., Muylkens, B., Thiry, E., Maes, P., Matthijnssens, J., 2014. Complete Genome Sequence of Equid Herpesvirus 3. Genome Announc 2, 12–13.

Slater, J., 2014. Equine Herpesviruses, in: Equine Infectious Diseases. Elsevier Inc., p. 151–168.e8.

Slater, J., 2007. Equine Herpesviruses, in: Equine Infectious Diseases. Elsevier Inc., pp. 134–153.

Slater, J.D., Lunn, D.P., Horohov, D.W., Antczak, D.F., Babiuk, L., Breathnach, C., Chang, Y.W., Davis-Poynter, N., Edington, N., Ellis, S., Foote, C., Goehring, L., Kohn, C.W., Kydd, J., Matsumura, T., Minke, J., Morley, P., Mumford, J., Neubauer, T., O'Callaghan, D., Osterrieder, K., Reed, S., Smith, K., Townsend, H., van der Meulen, K., Whalley, M., Wilson, W.D., 2006. Report of the equine herpesvirus-1 Havermeyer Workshop, San Gimignano, Tuscany, June 2004. Vet. Immunol. Immunopathol. 111, 3–13.

Smith, K., Borchers, K., 2001. A study of the pathogenesis of equid herpesvirus-1 (EHV-1) abortion by DNA in-situ hybridization. J Comp Pathol 125, 304–10.

Smith, K., Whitwell, K., Binns, M., Dolby, C., Hannant, A., Mumford, J., 1992. Abortion of virologically negative foetuses following experimental challenge of pregnant pony mares with equid herpesvirus 1. Equine Vet J. 24, 256–9.

Smith, K.C., Mumford, J.A., Lakhani, K., 1996. Comparison of equid herpesvirus-1 (EHV-1) vascular lesions in the early versus late pregnant equine uterus. J. Comp. Pathol. 114, 231–247.

Smith, K.C., Whitwell, K.E., Mumford, J.A., Gower, S.M., Hannant, J.P., Tearle, J.P., 1993. An immunohistological study of the uterus of mares following experimental infection by equid herpesvirus 1. Equine Vet. J. 25, 36–40.

Smith, K.L., Allen, G.P., Branscum, A.J., Frank Cook, R., Vickers, M.L., Timoney, P.J., Balasuriya, U.B.R., 2010. The increased prevalence of neuropathogenic strains of EHV-1 in equine abortions. Vet. Microbiol. 141, 5–11.

Spear, P.G., Longnecker, R., 2003. Herpesvirus entry: an update. J. Virol. 77, 10179–10185.

Steinman, R., Idoyaga, J., 2010. Features of the dendritic cell lineage. Immunol Rev 234, 5–17.

Steukers, L., Vandekerckhove, A.P., Van Den Broeck, W., Glorieux, S., Nauwynck, H.J., 2011. Comparative analysis of replication characteristics of BoHV-1 subtypes in bovine respiratory and genital mucosa explants: A phylogenetic enlightenment. Vet. Res. 42, 33.

Steukers, L., Weyers, S., Yang, X., Vandekerckhove, A., Glorieux, S., Cornelissen, M., Van Den Broeck, W., Temmerman, M., Nauwynck, H., 2014. Mimicking herpes simplex virus 1 and herpes simplex virus 2 mucosal behavior in a well characterized human genital organ culture. J. Infect. Dis. 210, 209–213.

Stringer, A., Lunn, D.P., Reid, S., 2015. Science in brief: Report on the first Havemeyer workshop on infectious diseases in working equids, Addis Ababa, Ethiopia, November 2013. Equine Vet. J. 47, 6–9.

Studdert, M.J., Simpson, T., Roizman, B., 1981. Differentiation of respiratory and abortigenic isolates of equine herpesvirus 1 by restriction endonucleases. Science (80-.). 214, 562–564.

Sukita, S., Furuse, M., Itoh, M., 2001. Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol 2, 285–93.

Swerczek, T.W., Dennis, S.M., 2006. Equine Herpesvirus Infections, in: Current Therapy in Large Animal Theriogenology: Second Edition. Elsevier Inc., pp. 176–180.

Tanzer, M.L., 2006. Current concepts of extracellular matrix. J. Orthop. Sci. 11, 326–331.

Tearle, J.P., Smith, K.C., Boyle, M.S., Binns, M.M., Livesay, G.J., Mumford, J.A., 1996. Replication of equid herpesvirus-1 (EHV-1) in the testes and epididymides of ponies and venereal shedding of infectious virus. J. Comp. Pathol. 115, 385–397.

Telford, E.A., Watson, M.S., Aird, H.C., Perry, J., Davison, A.., 1995. The DNA sequence of equine herpesvirus 2. J. Mol. Biol. 249, 520–528.

Telford, E.A., Watson, M.S., Aird, H.C., Perry, J., Davison, A.J., 1998. The DNA sequence of equine herpesvirus 4. J. Gen. Virol. 79, 1197–1203.

Telford, E.A.R., Studdert, M.J., Agius, C.T., Watson, M.S., Aird, H.C., Davison, A.J., 1993. Equine Herpesviruses 2 and 5 Are γ-Herpesviruses. Virology 195, 492–499.

Telford, E.A.R., Watson, M.S., McBride, K., Davison, A.J., 1992. The DNA sequence of equine herpesvirus-1. Virology 189, 304–316.

Tulinski, P., Fluit, A.C., van Putten, J.P.M., de Bruin, A., Glorieux, S., Wagenaar, J.A., Duim, B., 2013. An Ex Vivo Porcine Nasal Mucosa Explants Model to Study MRSA Colonization. PLoS One 8, 70–75.

USDA-APHIS, 2007. Equine herpesvirus myeloencephalopathy: a potential emerging disease. https://www.aphis.usda.gov/animal_health/emergingissues/downloads/ehv1final.pdf (accessed 7.7.16).

Vairo, S., Van Den Broeck, W., Favoreel, H., Scagliarini, A., Nauwynck, H., 2013. Development and use of a polarized equine upper respiratory tract mucosal explant system to study the early phase of pathogenesis of a European strain of equine arteritis virus. Vet. Res. 44, 1.

Van de Walle, G.R., Goupil, R., Wishon, C., Damiani, A., Perkins, G. a, Osterrieder, N., 2009. A single-nucleotide polymorphism in a herpesvirus DNA polymerase is sufficient to cause lethal neurological disease. J. Infect. Dis. 200, 20–25.

Van De Walle, G.R., May, M.L., Einem, J. Von, Osterrieder, N., Walle, G.R. Van De, May, M.L., Sukhumavasi, W., Einem, J. Von, Osterrieder, N., 2007. Herpesvirus Chemokine-Binding Glycoprotein G (gG) Efficiently Inhibits Neutrophil Chemotaxis In Vitro and In Vivo. J. Immunol. 179, 4161–4169.

Van de Walle, G.R., Peters, S.T., VanderVen, B.C., O'Callaghan, D.J., Osterrieder, N., 2008. Equine herpesvirus 1 entry via endocytosis is facilitated by alphaV integrins and an RSD motif in glycoprotein D. J. Virol. 82, 11859–68.

Van der Meulen, K., Caij, A., Smets, K., Nauwynck, H., 2006. Equine coital exanthema in mare

in Belgium. Vlaams Diergeneeskd. Tijdschr. 75, 286–289.

Van Maanen, C., 2002. Equine herpesvirus 1 and 4 infections: An update. Vet. Q. 24, 58–78.

Vandekerckhove, A., Glorieux, S., Broeck, W. Van Den, Gryspeerdt, A., van der Meulen, K.M., Nauwynck, H.J., 2009. In vitro culture of equine respiratory mucosa explants. Vet. J. 181, 280–287.

Vandekerckhove, A.P., Glorieux, S., Gryspeerdt, A.C., Steukers, L., Duchateau, L., Osterrieder, N., Van De Walle, G.R., Nauwynck, H.J., 2010. Replication kinetics of neurovirulent versus non-neurovirulent equine herpesvirus type 1 strains in equine nasal mucosal explants. J. Gen. Virol. 91, 2019–2028.

Vandekerckhove, A.P., Glorieux, S., Gryspeerdt, A.C., Steukers, L., Van Doorsselaere, J., Osterrieder, N., Van de Walle, G.R., Nauwynck, H.J., 2011. Equine alphaherpesviruses (EHV-1 and EHV-4) differ in their efficiency to infect mononuclear cells during early steps of infection in nasal mucosal explants. Vet. Microbiol. 152, 21–28.

Vareille, M., Kieninger, E., Edwards, M.R., Regamey, N., 2011. The airway epithelium: Soldier in the fight against respiratory viruses. Clin. Microbiol. Rev. 24, 210–229.

Varol, C., Mildner, A., Jung, S., 2015. Macrophages: development and tissue specialization. Annu Rev Immunol 33, 643–675.

Walter, J., Balzer, H.J., Seeh, C., Fey, K., Bleul, U., Osterrieder, N., 2012. Venereal Shedding of Equid Herpesvirus-1 (EHV-1) in Naturally Infected Stallions. J. Vet. Intern. Med. 26, 1500–1504.

Wang, L., Raidal, S.L., Pizzirani, A., Wilcox, G.E., 2007. Detection of respiratory herpesviruses in foals and adult horses determined by nested multiplex PCR. Vet. Microbiol. 121, 18–28.

Wellington, J.E., Love, D.N., Whalley, J.M., 1996. Evidence for involvement of equine herpesvirus 1 glycoprotein B in cell-cell fusion. Arch. Virol 141, 167–75.

Whitwell, K., Blunden, A., 1992. Pathological findings in horses dying during an outbreak of the paralytic form of Equid herpesvirus type 1 (EHV-1) infection. Equine Vet J. 24, 13–9.

Williams, K.J., Maes, R., Del Piero, F., Lim, A., Wise, A., Bolin, D., Caswell, J., Jackson, C., Robinson, N., Derksen, F., 2007. Equine multinodular pulmonary fibrosis: A newly recognized herpesvirus-associated fibrotic lung disease. Vet Pathol. 44, 849–62.

Wilson, W.D., 1997. Equine herpesvirus 1 myeloencephalopathy. Vet. Clin. North Am. Equine Pract. 13, 53–72.

Wilsterman, S., Soboll-Hussey, G., Lunn, D.P., Ashton, L. V., Callan, R.J., Hussey, S.B., Rao, S., Goehring, L.S., 2011. Equine herpesvirus-1 infected peripheral blood mononuclear cell subpopulations during viremia. Vet. Microbiol. 149, 40–47.

Wohlsein, P., Lehmbecker, A., Spitzbarth, I., Algermissen, D., Baumgärtner, W., Böer, M., Kummrow, M., Haas, L., Grummer, B., 2011. Fatal epizootic equine herpesvirus 1 infections in new and unnatural hosts. Vet. Microbiol. 149, 456–460.

Wood, J., Smith, K.C., Daly, J.M., Newton, J.R., 2007. Viral infections of the equine respiratory tract, Equine Respiratory Medicine and Surgery. Elsevier Ltd.

CHAPTER 2

AIMS OF THE THESIS

Equine herpesviruses (EHVs) are distributed worldwide and are responsible for a variety of diseases in equids. The mucosal surface of the respiratory tract represents the principal portal of entry for EHVs. EHV-1 primarily replicates in the epithelial cells of the upper respiratory tract, after which it can spread throughout the body via a cell-associated viremia to the endothelial cells of the pregnant uterus, the central nervous system or the eye, resulting in abortion, myeloencephalopathy, or chorioretinopathy. Currently available vaccines against EHV-1 and EHV-4 do not reliably prevent infection, development of viremia or establishment of latency. To date, no specific therapy is effective against EHV infections. Thus, early recognition of the disease is very crucial for the implementation of management practices that decrease the risk of exposure of susceptible equids. Several epidemiological studies have been conducted on EHVs, but many data gaps exist and more investigation needs to be done to better understand the epidemiology of the disease in the different geographical settings and unvaccinated equine population.

Mucosal tissues can differ significantly when it comes to structural barriers, microenvironment, and the composition of resident immune cells. This unique characteristic of different mucosal tissues influences the efficiency of replication and invasion of viruses. So far, studies have been conducted on the invasion characteristics of EHV-1 and EHV-4 in the equine respiratory mucosa. Initial infection starts with replication in the epithelial cells of the upper respiratory tract. EHV-4 infection is mostly limited to the respiratory tract, whereas EHV-1 is able to spread rapidly beyond the respiratory tract to become a systemic infection. In previous studies, the presence of EHV-1 has been reported in testis and semen of infected stallions, but their transmission through the vaginal mucosa is not known. Therefore, it is very interesting to evaluate the efficiency of EHV-1 invasion through the vaginal mucosa. Further unraveling of EHV-1 entry route via the vaginal mucosa may provide new insights into the pathogenesis of EHV-1. Infection with EHV-3 occurs through direct skin-to-skin contact during coitus or through secretions containing live virus, however, the underlying pathogenesis remains poorly understood. Thus, it is important to elucidate the viral behavior during the early stage of EHV-3 infection. A thorough understanding of EHV mucosal invasion mechanisms will provide new insights towards the development of alternative preventive and curative strategies.

Therefore, the first general aim of this doctoral thesis was to gain a better understanding of EHVs epidemiology. A second general aim was to obtain more insights into the invasion characteristics of two specifically chosen EHV types, namely EHV-1 and -3.

The specific aims of this thesis were:

- To isolate and genetically characterize EHV-1 from equids clinically affected with herpesvirus myeloencephalopathy during outbreaks in the Northern part of Ethiopia between 2011 and 2013 (Chapter 3.1).
- To determine the prevalence of EHV-1, -2, -4, and -5 in Ethiopian equids with and without clinical signs of respiratory disease and genetically characterize EHV-2 and -5 strains. For this purpose, a large cohort of equids was sampled in the Northern part of Ethiopia (Chapter 3.2).
- To evaluate the replication kinetics and invasion characteristics of the neuropathogenic and abortigenic strains of EHV-1 and EHV-3 in the respiratory and genital mucosae using *ex vivo* tissue cultures (Chapter 4).

CHAPTER 3

EQUINE HERPESVIRUSES IN ETHIOPIAN EQUIDS

CHAPTER 3.1

Equine herpesvirus 1 myeloencephalopathy, an emerging threat of working equids in Ethiopia

Adapted from

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Abstract

Although equine herpesvirus myeloencephalopathy (EHM) is a sporadic and relatively uncommon manifestation of equine herpesvirus-1 (EHV-1), it has the potential for causing devastating outbreaks in horses. Up till now, there were no reported EHM outbreaks in donkeys and mules. The present study describes the isolation and molecular characterization of EHV-1 from clinically EHM affected horses (n = 6), mules (n = 3), and donkeys (n = 82) in Ethiopia during outbreaks from May 2011 to December 2013. The incidence of EHM cases was higher from April to mid-June. However, EHM in donkeys was more severe and regularly death occurred after a short period of neurological signs. EHM-affected equids mainly observed over 3 years of age and equids within the age ranged from 7 - 10 years (n = 51; 56.0%) were mostly affected. Females (n = 58; 63.7%) were more affected than males. The incidence of neuropathogenic (D_{752}) and non-neuropathogenic (N_{752}) variants of EHV-1 from EHM affected equids in Ethiopia was assessed by sequencing the DNA polymerase gene (ORF30) of the EHV-1 isolates. The results indicated that from the total of 91 clinically affected equids, 90 (98.9%) of them had an ORF30 D_{752} genotype. An ORF30 N_{752} variant was only found in 1 donkey. Analysis of ORF68 as grouping marker for geographical differences showed that the Ethiopian EHV-1 isolates belong to geographical group 4. Due to the fatal nature of EHV-1 in donkeys, it would be interesting to examine the pathogenesis of EHM in this species. At present, there is no vaccine available in Ethiopia, and therefore outbreaks of EHV-1 should be controlled by proper management adaptations. In addition, it is important to test the efficacy of the commercial vaccines not only in horses but also in donkeys and mules.

Introduction

Equine herpesvirus 1 (EHV-1) is an important, ubiquitous viral pathogen in most equine populations throughout the world, which causes disease and extensive economic losses during frequent outbreaks of respiratory disease, abortion, neonatal foal death, myeloencephalopathy, and chorioretinopathy (Allen et al., 2004; Kydd et al., 2006; Hussey et al., 2013). EHV-1 is a member of the *Alphaherpesvirinae* of the family *Herpesviridae*, with a 150 kilobases double stranded DNA genome, consisting of 76 unique open reading frames (ORF) (Telford et al., 1992).

EHV-1 establishes a lifelong infection (latency) in a high percentage of equids following exposure to the virus. Infections are particularly common in young performance horses and typically result in the establishment of a latent infection within the first months of life, with subsequent viral reactivation causing clinical disease and viral shedding during periods of stress (Goehring and Lunn, 2008). Outbreaks of EHV-1 neurological problems are thought to be initiated either by viral reactivation of latent EHV-1 (Allen and Timoney, 2007) or by virus transmission after a recent history of abortion or respiratory disease in an affected horse population (Pusterla et al., 2009).

In recent years, there has been an unexpected increase in the incidence of equine herpesvirus neurological disease, equine herpesvirus myeloencephalopathy (EHM) (Perkins et al., 2009; Vissani et al., 2009; Fritsche and Borchers, 2010; Pronost et al., 2010; Smith et al., 2010). This EHM is classified as a potentially emerging disease by the US Department of Agriculture (USDA-APHIS, 2007). Studies suggested that EHM is strongly associated with a single nucleotide polymorphism (SNP) at position 2254 in the EHV-1 DNA polymerase gene ORF30 (Nugent et al., 2006; Goodman et al., 2007; Perkins et al., 2009). Neuropathogenic EHV-1 strains have been reported to replicate more efficiently and achieve 10-fold higher levels of leukocyte-associated viremia as compared to horses infected with non-neurovirulent strains (Allen and Breathnach, 2006; Goodman et al., 2007; Van de Walle et al., 2009). A direct *in vivo* and *ex vivo* comparison of European EHV-1 strains has shown that more leukocytes are attracted and become infected in the nasal mucosa with the

neurovirulent strains compared with the non-neurovirulent strains (Gryspeerdt et al., 2010; Vandekerckhove et al., 2010).

Infections with EHV-1 have been identified in other members of the family *Equidae* than horses, including mules and donkeys, which establish typically silent infections and serve as reservoir hosts (Van Maanen, 2002; Pusterla et al., 2012). Although all breeds of horses are susceptible to the neurologic form of EHV-1 infection, EHM from clinically affected donkeys and mules are poorly reported.

According to a livestock survey, there are about 6.44 million donkeys, 1.96 million horses, and 373,519 mules recorded in Ethiopia (CSA, 2011). It has the largest equine population, probably with the highest density per square kilometer in the world (Feseha, 1998). These equids have an essential role in the livelihood of their owners and are mainly used for transportation, draught power, and other purposes. Recently, the Ministry of Agriculture of Ethiopia frequently received reports of outbreaks suspected of being induced by EHV-1 (personal communications). Substantial numbers of equids (horses, mules, and donkeys) have died after showing clinical signs such as depression, anorexia, ataxia, and lameness. Because the etiology was never identified, the epidemiological situation of EHV-1 in Ethiopia was totally unknown. This study was designed for the isolation of EHV-1 and molecular characterization of the isolates from clinically affected horses, mules, and donkeys in Ethiopia.

Materials and methods

Outbreak investigation

This study was conducted from May 2011 to December 2013 in the northern part of Ethiopia, especially in Amhara Regional State, an equine-dense population, where an outbreak of neurological problems was reported (Figure 1). Individual cases were clinically investigated. The following parameters were included: fever (rectal temperature $\geq 38^{o}$C for donkeys and $\geq 38.5^{o}$C for horses and mules), respiratory problems, edema at the ventral region of the abdomen/limbs, abortion, neonatal foal death or neurological signs. Clinical and

epidemiological information were gathered and specimens were collected for EHV-1 diagnosis.

Figure 1: Map of Ethiopia that shows the districts where EHV-1 outbreaks were reported. 1: Dembia, 2: Chilga, 3: Dera, 4: Bahir Dar Zuria, 5: Mecha, 6: South Achefer, 7: Dangla, 8: Awabel, 9: Angolelana Tera, 10: Menz Keya Gebreal

Sampling and processing protocol

Nasopharyngeal swabs from apparently sick equids or tissue samples (spleen, thymus, liver, and/or lungs) from recently deceased equids were collected and transported in viral transport medium: phosphate buffered saline (pH of 7.2 - 7.6) with antibiotics. Whole blood samples were also collected from living animals using an EDTA vacutainer tube. The peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation using Ficoll-Paque PLUS (GE Health Care, Life Sciences). Each sample was labeled using a unique code consisting of the country (ETH), year of the outbreak (Y), and identification number (N). All collected samples were immediately placed in a cooler containing ice for

transport to the National Veterinary Institute, Ethiopia. Samples were kept at -70^{o}C until further processing.

Virus isolation

Virus isolation from nasopharyngeal swabs, tissue suspension (10% w/v), and PBMCs were performed on confluent rabbit kidney (RK13) cells cultured in Dulbecco's modified Eagle medium (DMEM) at 37^{o}C in an atmosphere containing 5% CO_2. The cells were monitored daily microscopically for the appearance of a cytopathic effect (CPE) for 7 days. If CPE was not detected, the cells were passaged blindly three times before the samples were declared negative.

Nucleic acid extraction, PCR amplification, and sequencing

EHV-1 DNA was extracted from 200µl of nasopharyngeal secretions, whole blood samples, and cell culture supernatant using a QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions with a final DNA elution volume of 60µl. PCR assay was performed using Agilent's Herculase II fusion DNA polymerase (Agilent Technologies, USA). DNA amplification was carried out in a final volume of 25µl PCR reaction mixtures containing 5µl of 5x Herculase II reaction buffer, 0.5µl of 25 mM each deoxynucleoside triphosphate (dNTP) mix, 0.5µl Herculase II fusion DNA polymerase, 1µl of each forward and reverse primers, 2.5µl of dimethyl sulfoxide (DMSO), 2.5µl of template DNA, and 12µl of nuclease-free water.

The clinical diagnosis of EHV-1 was confirmed by PCR using specific primers (Table 1) targeting EHV-1 glycoprotein B and further sequence analysis. This protein contains highly specific regions that allow discrimination between closely related EHV-1 and EHV-4 (Wagner et al., 1992; Borchers and Slater, 1993; Kirisawa et al., 1993).

PCR amplification and subsequent sequencing of the DNA polymerase gene (ORF30) were performed using specific primers (Table 1) to amplify a region of 466bp, according to Goodman et al. (2007) to determine the prevalence of neurological and non-neurological variants (D_{752}/N_{752}) of EHV-1 from EHM affected equids in Ethiopia. The region of interest of ORF30 was amplified with an initial denaturation step of 95^{o}C for 15 min, followed by

34 cycles of denaturation at 94^{o}C for 1 min, annealing at 55.5^{o}C for 1 min, extension at 72^{o}C for 1 min, and a final extension at 72^{o}C for 10 min.

A 645bp region of ORF68 was also amplified using specific primers (see Table 1) and sequenced as described by Nugent et al. (2006). This sequence allows grouping of EHV-1 isolates. The PCR consisted of an initial denaturation step of 94^{o}C for 4 min, followed by 34 cycles of denaturation at 94°C for 30 seconds, primer annealing for 1 min at 48^{o}C, elongation 72^{o}C for 2 min, and a final extension at 72^{o}C for 10 min.

Table 1: Primers used for amplification and sequencing of specific regions of the genome of EHV-1.

Region	PCR primers	Use	Size	References
ORF30	Fw: 5'-GCTACTTCTGAAAACGGAGGC-3'	PCR/Seq	466bp	Goodman et al. (2007)
	Rv: 5'-TATCCTCAGACACG GCAACA-3'	PCR/Seq		
ORF68	Fw: 5′- AGCATTGCCAAACAGTTCC-3'	PCR/Seq	645bp	Nugent et al. (2006)
	Rv: 5′-CAAGAAACCACTGCTCAACC-3'	PCR/Seq		
	S1: 5'-GAAGATAGAATGGGTGTGAG-3	Seq		
gB	Fw: 5'- GCGTTATAGCTATCACGTCC-3'	PCR/Seq	190bp	Kirisawa et al. (1993)
	Rv: 5'- ATACGATCACATCCAATCCC- 3'	PCR/Seq		

Amplicons of EHV-1 were detected by agarose gel electrophoresis and visualized under UV light after ethidium bromide staining. PCR products were purified using NucleoSpin®Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co. KG, Germany) in accordance with the manufacturer's instructions. The purified PCR products were sequenced by GATC Biotech, Germany, (ABI 3730XL Sanger sequencing platform) with sequencing primers. The genomic sequence of the parental EHV-1 strain Ab4, GenBank accession number AY665713, was used for comparison of sequences.

Sequences were assembled and edited using the DNASTAR® software program, SeqMan Pro (version 12: DNASTAR, Inc.). Following sequence assembly, multiple alignments of

homologous sequences were analyzed using the ClustalW Multiple alignment programs implemented in BioEdit software version 7. A phylogenetic tree was constructed by the unweighted pair group method with arithmetic mean (UPGMA) using MEGA 6.06 software program. EHV-1 ORF68 genes of representative reference strains were retrieved from GenBank.

Results

Description of EHV-1 outbreaks

During the period from May 2011 to December 2013, a total of 14 EHV-1 outbreaks in 10 districts of Ethiopia were recorded. A total of 91 equids that showed clinical signs suggestive of EHM were included in this study, of which 82 of them were donkeys, 6 horses, and 3 mules (Table 2). Outbreaks of EHM occurred during all seasons of the year, but a higher incidence of the disease was recorded from April to mid-June (data not shown).

Table 2: Number of equids clinically affected by EHM during different years

Sampling year	Number of equids affected			
	Horse	Mule	Donkey	Total
2011	0	0	33	33
2012	0	0	7	7
2013	6	3	42	51
2011 - 2013	6	3	82	91

In each outbreak, equids were carefully examined for the presence of characteristic clinical signs of EHM. Of the 91 EHM affected equids, 38 (41.8%) equids had raised rectal temperature, 7 (7.7%) had edema in the lower abdomen and/or hind limbs, 91 (100%) had ataxia and paresis (staggering movement), 1 (1.1%) had paralysis, and 1 (1.1%) had urinary incontinence. Furthermore, 11 (12.1%) equids had respiratory signs characterized by serous to mucopurulent nasal discharge (Table 3). There were no visible differences in the clinical presentation of EHM among horses, mules, and donkeys. However, However, EHM in

donkeys was more severe and regularly death occurred after a short period of neurological signs.

Table 3: Clinical data observed in EHM-affected horses, mules, and donkeys

Clinical signs	Number of equids demonstrating the clinical signs (%)			
	Horse (n = 6)	Mule (n = 3)	Donkey (n = 82)	Total (n = 91)
Fever	2 (33.3%)	1 (33.3%)	35 (42.7%)	38 (41.8%)
Nasal discharge	2 (33.3%)	0 (0.0%)	9 (11.0%)	11 (12.1%)
Edema on lower abdomen/limb	1 (16.7%)	0 (0.0%)	6 (7.3%)	7 (7.7%)
Urinary incontinence	1 (16.7%)	0 (0.0%)	0 (0.0%)	1 (1.1%)
Ataxia and paresis	6 (100%)	3 (100%)	82 (100%)	91 (100.0%)
Paralysis	1 (16.7%)	0 (0.0%)	0 (0.0%)	1 (1.1%)

The age of the EHM affected equids ranged from 2½ - 14 years (median 9 years), with 51 (56%) equids at the age of 7 - 10 years. Only 2 donkeys were clinically affected at the age of less than 3 years. A higher proportion of EHM was recorded in females (58; 63.7%) than males (33; 36.3%) (Table 4).

EHV-1 isolation and PCR detection

EHV-1 was successfully isolated in confluent RK-13 cells from nasopharyngeal swabs, tissue samples (liver, lungs, thymus, and spleen), and PBMCs of equids showing clinical signs of EHM. Characteristic EHV-1 CPE (rounding, increase in refractility, and detachment of cells) were observed in the RK-13 monolayers. All isolates from EHM-affected equids were PCR positive using specific primers of EHV-1 gB, which allow the discrimination between EHV-1 and EHV- 4. During the investigation period, a total of 12 abortion cases were recorded and tissue samples from both aborted fetuses and placentas and whole blood samples were collected and examined for EHV-1 by isolation and PCR. All samples were negative.

Table 4: Intrinsic host risk factors associated with EHM observed within horses, mules, and donkeys

Risk factors	Number of equids demonstrating EHM (%)			
	Horse (n = 6)	Mule (n = 3)	Donkey (n = 82)	Total (n = 91)
Age				
< 3 years	0 (0.0%)	0 (0.0%)	2 (2.4%)	2 (2.2%)
4-6 years	1 (16.7%)	1 (33.3%)	19 (23.2%)	21 (23.1%)
7-10 years	5 (83.3%)	1 (33.3%)	45 (54.9%)	51 (56.0%)
> 10 years	0 (0.0%)	1 (33.3%)	16 (19.5%)	17 (18.7%)
Gender				
Male	1 (1.1%)	0 (0.0%)	32 (39.0%)	33 (36.3%)
Female	5 (83.3%)	3 (100%)	50 (61.0)	58 (63.7%)

EHV-1 ORF30 sequence

EHV-1 polymerase gene (ORF30) was PCR amplified and sequenced to estimate the prevalence of neurological (D_{752}) and non-neurological (N_{752}) variants of the virus. Of the 91 clinically affected equids with neurological disorders, the ORF30 D_{752} variant was detected in 90 (98.9%) equids, of which 81 (98.8%) of them were donkeys, 3 (100%) mules, and 6 (100%) horses. An ORF30 N_{752} variant was found only in 1 (1.1%) donkey (Table 5).

Table 5: Prevalence of N_{752} and D_{752} in the ORF30 gene of EHV-1 isolates from horses, mules, and donkeys

Genotype	Number of EHV-1 isolates (%)			
	Horse (n = 6)	Mule (n = 3)	Donkey (n = 82)	Total (n = 91)
ORF30 N_{752}	-	-	1 (1.22%)	1 (1.1%)
ORF30 D_{752}	6 (100%)	3 (100%)	81 (98.8%)	90 (98.9%)

EHV-1 ORF68 sequence

EHV-1 ORF68 gene (nucleotides sequence 125,387 - 125,945 in EHV-1 Ab4, GenBank reference AY665713) was sequenced for all isolates and used to group the Ethiopian EHV-1 isolates for geographical differences and compared to isolates from European countries, North and South America, and Australia according to Nugent et al. (2006). ORF68 was identical for all Ethiopian EHV-1 isolates and was categorized, according to the proposed classification in geographical group four (Figure 2). The ORF68 nucleotide sequences of the Ethiopian EHV-1 isolates were identical and therefore, a single nucleotide sequence from each species of equids was submitted to GenBank under accession numbers KP765722 from a horse, KP765723 from a mule, and KP765724 from a donkey.

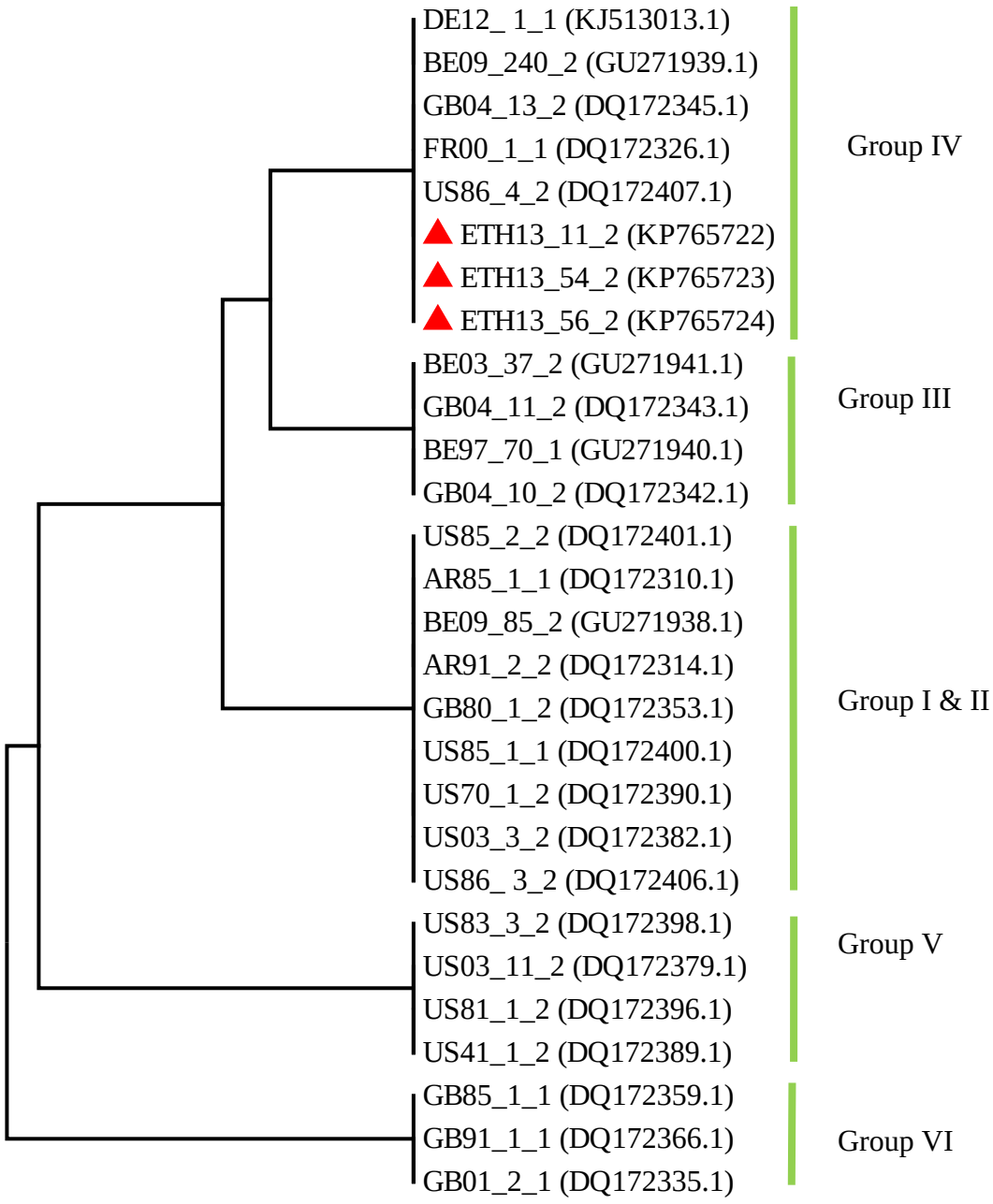

Figure 2. Phylogenetic tree constructed by the UPGMA method using EHV-1 ORF68 sequences. The first two or three letters indicate the country of origin, followed by the year of outbreak, unique identification number, and then the clinical outcome: non-neuropathogenic (1) or neuropathogenic (2). The Ethiopian isolates have identical ORF68 sequences and therefore, one sequence from the isolates of each equid species is used for the phylogenetic tree. ETH13_11_2 (KP765722) is an isolate from a horse; ETH13_54_2

(KP765723) is an isolate from a mule; ETH13_56_2 (KP765724) is an isolate from a donkey. GenBank accession numbers are indicated in between parentheses.

Discussion

EHV-1 is the most prevalent virus in horse populations throughout the world that causes substantial equine losses. In this study, we describe the isolation and molecular characterization of EHV-1 from outbreaks of EHM in horses, mules, and donkeys in Ethiopia. These EHM outbreaks in Ethiopia are considered as emerging in working equids based on the following facts: (i) this is the first report of EHV-1-associated problems in the country, (ii) the outbreaks are increasing in time, (iii) large number of donkeys, horses, and mules are clinically affected, and (iv) the disease is spreading fast to different parts of Ethiopia. Prior to 2009, the presence of EHV-1 in equine populations had not been reported in Ethiopia (personal communication Ministry of Agriculture, Ethiopia). Since then, frequent and widespread EHV-1 outbreaks especially EHM have been described. During the period of 2011 to 2013, 14 EHM outbreaks in 10 districts of the country were recorded. In Ethiopia, the import of equids from other countries has not been documented. Therefore, the possible source of these EHV-1 outbreaks in Ethiopia is unknown. In recent years, outbreaks of EHM have been reported with increasing frequency and severity in most parts of the world (McCartan et al., 1995; van Maanen et al., 2001; USDA-APHIS, 2007; Damiani et al., 2014). Different risk factors are involved in the severity of the disease associated with EHV-1 infection, including (i) the age and physical condition of the host, (ii) whether the infection is primary, a re-infection, or a reactivation of latent virus, (iii) the immune status of the host, and (iv) the pathogenicity of the strain involved (Allen et al., 2004; Allen, 2008).

In this study, we observed that the EHM related clinical signs in donkeys were more severe (often fatal) than in horses and mules. This difference in severity might be associated with the host factor. Ethiopian donkeys might be highly susceptible to EHM infection. Barbic et al. (2012) reported a significant influence of breed on the clinical manifestation of neuropathogenic EHV-1 infections.

In the present study, a total of 91 clinically EHM affected equids were recorded, of which 82 of them were donkeys, 6 horses, and 3 mules. EHM affected a larger proportion of donkeys than horses and mules. The present report of a clinical outcome of EHV-1 infections in donkeys and mules are in contrast to previous reports. Van Maanen (2002) stated that donkeys are an alternative host for EHV-1 and serve as a silent reservoir for infections in horses. Pusterla et al. (2012) reported that mules are protected against the development of neurological signs and that they are silent shedders of EHV-1 during an EHM outbreak among horses. To our knowledge, this is the first report of EHM affected donkeys and mules. The difference in the clinical outcome of EHV-1 infections in donkeys and mules might be explained by the fact that equids in Ethiopia are subjected to several stress factors such as heavy workload, traveling long distances, poor nutritional state, heavy parasite burden, and concurrent diseases. Another reason could be a difference in the pathogenic potential of EHV-1 strains. Horses, mules, and donkeys are sharing common paddocks, grazing pastures, and drinking places which might have led to trans-species transmission of the virus among horses, mules, and donkeys.

Outbreaks of EHM were reported during all seasons of the year, but a higher incidence of EHM was observed from April till mid-June. Strong seasonal clustering of EHM outbreaks was described previously by Goehring et al. (2006), with all outbreaks occurring between mid-November and mid-May. This seasonal clustering of EHM outbreaks in late autumn, winter, and spring are associated with close confinement of equids (Lunn et al., 2009; Kydd et al., 2012). In Ethiopia this temporal clustering of EHM outbreaks is mainly associated with feed shortage, leading to a grouping of the animals in small pastures and paddocks and increasing stress.

An increased body temperature was detected in 38 (41.8%) equids. The body temperature was measured during sampling from clinically affected equids. Previous studies on naturally occurring outbreaks of EHM indicated that fever occurs days before the onset of neurological signs, but is often absent during the neurological disorders (Henninger et al., 2007). Gryspeerdt et al. (2011) also reported the rapid occurrence of ataxia and paralysis immediately after the disappearance of fever. Therefore, in this study, the number of febrile

equids was higher than recorded before. The rise of body temperature should be considered as an early warning for EHM, which is in agreement with other studies.

Mainly equids of 3 - 14 years (median 9 years) were clinically affected. Only 2 donkeys were showing clinical signs at the age of less than three years. This is consistent with previous studies. Goehring et al. (2006) reported that EHM was largely restricted to horses over 3 years of age. Allen and Breathnach (2006) observed a failure to develop neurological disease when yearling horses were experimentally infected with neuropathogenic strains of EHV-1. Although we clearly demonstrated that clinical EHM was largely restricted to equids over three years of age, the association of clinical manifestation of EHM and immunity remain to be determined. The highest proportion of equids was affected at the age ranging from 7 - 10 years. This is in line with other studies where the risk of EHM outbreaks was the highest in middle-aged or older horses (Barbic et al., 2012; reviewed by Pusterla and Hussey et al., 2014). In the present study, higher proportions of females were affected than males. This is also in line with what has been observed in other studies, where females in naturally occurring EHM are at higher risk of developing neurological problems (Goehring et al., 2006; and Allen, 2008). At present, there is no clear explanation why both age and gender are related to the clinical outcome of an EHV-1 infection.

Molecular epidemiological studies of EHV-1 have shown that a SNP in the DNA polymerase gene (ORF30), the change of adenine (A) to guanine (G) at nucleotide position of 2254 resulting in an asparagine (N) to aspartic acid (D) change at amino acid position of 752, is strongly associated with outbreaks of highly lethal neurological disease in horses (Nugent et al., 2006; Van de Walle et al., 2009). Goodman et al. (2007) revealed that the point mutation of the DNA polymerase EHV-1 gene, from G_{2254} to A_{2254} encoding N_{752} instead of D_{752} variants, results in a reduced virus replication in PBMCs, altered tropism for leukocyte subsets, and absence of neurological disease. In Ethiopia, 98.9% (90 out of 91) of EHV-1 isolates were identified as neuropathogenic variants (D_{752}), of which 81 (98.8%) of them were from donkeys, 6 (100%) from horses, and 3 (100%) from mules. Only 1 (1.22%) donkey, that displayed EHM, was infected with a non-neuropathogenic variant (N_{752}). In general, 15 to 25% of EHM outbreaks are induced by non-neuropathogenic (N_{752}) variants (Lunn et al., 2009). In this study, the prevalence of non-neuropathogenic variants from EHM

affected equids is far less than other reports in France (56%; 9/16) (Pronost et al., 2010), in Argentina (50%; 2/4) (Vissani et al., 2009), and in North America (24%; 5/21) (Perkins et al., 2009). The failure of EHV-1 detection from cases of abortion and the highly prevalence of neuropathogenic variants in EHM affected equids indicated that neuropathogenic variants are the main strains of EHV-1 circulating in equine populations in Ethiopia. To the author's knowledge, EHM outbreaks were not reported earlier in the African continent.

ORF68 of EHV-1, homologous to herpes simplex virus type-1 Us2, has the highest sequence variation rate (2%) and is used as a grouping marker and provide evidence for geographical restriction of certain genotypes (Nugent et al., 2006). Based on mutations at nucleotide positions 336, 344, 629, 710, 713, 719, 731-740 and 755, strains from North America, South America, Europe, and Australia were classified into six geographical groups. The present study showed that the Ethiopian EHV-1 strains are assigned to group four. In this group, strains from the USA and European countries such as Germany, Belgium, Great Britain, and France were present. This indicates that in one way or another this virus entered Ethiopia in the past, leading to the current problems.

In conclusion, EHV-1 has been isolated and genetically characterized for the first time from EHM outbreaks in working equids of Ethiopia. It documents that donkeys and mules, besides horses, are clinically affected by the neurological form of EHV-1. The neuropathogenic variant of EHV-1 was mainly responsible for the EHM outbreaks and all the Ethiopian EHV-1 isolates were allocated to geographical group 4. EHM outbreaks have been the most prevalent clinical outcome of the EHV-1-associated diseases and caused devastating losses of equids especially in donkeys. Based on the relatively fast course and fatal nature of the EHV-1 infection in donkeys, it is worth studying the pathogenesis of EHM in donkeys both *in vivo* (*ex vivo*) and *in vitro*. Because available vaccines have never been evaluated in donkeys and mules, it is not clear to what degree they may give protection in these equid species. More work is needed to get an answer to this question.

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References

Allen, G.P., 2008: Risk factors for the development of neurologic disease after experimental exposure to equine herpesvirus - 1 in horses. Am J Vet Res. 69, 1595-1600.

Allen, G.P., Breathnach, C.C., 2006: Quantification by real-time PCR of the magnitude and duration of leucocyte-associated viraemia in horses infected with neuropathogenic vs. non-neuropathogenic strains of EHV-1. Equine Vet. J. 38, 252 - 257.

Allen, G.P., Timoney, P.J., 2007: Recent advances in our understanding of equine herpesvirus - 1 (EHV-1) myeloencaphalopathy. In: 107th annual meeting of the United States Animal Health Association, pp. 373 - 380.

Allen, G.P., Kydd, J.H., Slater, J.D., Smith, K.C., 2004: Equid herpesvirus - 1 and equid herpesvirus - 4 infections. In: Coetzer J.A.W, and R.C. Tustin (eds), Infectious Diseases of Livestock. 2nd edn. pp. 829 – 859. Oxford University Press, Cape Town.

Barbic, L., Lojkic, I., Stevanovic, V., Bedekovic, T., Staresina, V., Lemo, N., Lojkic, M., Madic, J., 2012: Two outbreaks of neuropathogenic equine herpesvirus type - 1 with breed-dependent clinical signs. Vet Rec. 170, 227.

Borchers, K., Slater, J., 1993: A nested PCR for the detection and differentiation of EHV-1 and EHV-4. J Virol Methods, 45, 331 - 336.

Central Statistical Authority (CSA), 2013: Federal democratic republic of Ethiopia agricultural sample survey 2012/13. Report on livestock and livestock characteristics (private peasant holdings), Statistical Bulletin, volume II, Addis Ababa, Ethiopia.

Damiani, A.M., de Vries, M., Reimers, G., Winkler, S., Osterrieder, N., 2014: A severe equine herpesvirus type 1 (EHV-1) abortion outbreak caused by a neuropathogenic strain at a breeding farm in northern Germany. Vet Microbiol. 172, 555 - 562.

Feseha, G., 1998: Proceeding on Equine Infectious Diseases, 8th International conference on Equine Infectious Diseases. Dubai, pp. 318-319.

Fritsche, A.K., Borchers, K., 2010: Detection of neuropathogenic strains of equid herpesvirus - 1 (EHV-1) associated with abortions in Germany. Vet. Microbiol. 147, 176 - 180.

Goehring, L.S., Lunn, D.P., 2008: Equine herpesvirus myeloencephalopathy. In: Robinson, N.E., and K. Sprayberry (eds), Current Therapy in Equine Medicine, 6th edn. pp., 177 - 181. St. Louis, Saunders.

Goehring, L.S., Van Winden, S.C., Van Maanen, C., Sloet Van Oldruitenborgh-Oosterbaan, M.M., 2006: Equine herpesvirus type - 1 associated myeloencephalopathy in the Netherlands: a four-year retrospective study (1999 - 2003). J Vet Intern Med. 20, 601 - 607.

Goodman, L.B., Loregian, A., Perkins, G.A., Nugent, J., Buckles, E.L., Mercorelli, B., Kydd, J.H., Palu, G., Smith, K.C., Osterrieder, N., Davis-Poynter, N., 2007: A point mutation in a herpesvirus polymerase determines neuropathogenicity. PLoS Pathog. 3, 4969 - 4978.

Gryspeerdt, A., Vandekerckhove, A., Doorsselaere, J.V., Van de Walle, G.R., Nauwynck, H.J., 2011: Description of an unusually large outbreak of nervous system disorders caused by equine herpesvirus - 1 (EHV-1) in 2009 in Belgium. Vlaams Diergen Tijds. 80, 147 - 153.

Gryspeerdt, A.C., Vandekerckhove, A.P., Garré, B., Barbé, F., Van de Walle, G.R., Nauwynck, H.J., 2010: Differences in replication kinetics and cell tropism between neurovirulent and non-neurovirulent EHV-1 strains during the acute phase of infection in horses. Vet Microbiol. 142, 242 - 253.

Henninger, R.W., Reed, S.M., Saville, W.J., Allen, G.P., Hass, G.F., Kohn, C.W., Sofaly, C., 2007: Outbreak of neurologic disease caused by equine herpesvirus - 1 at a University Equestrian Center. J Vet Intern Med. 21: 157 - 165.

Hussey, S.G., Goehring, S.L., Lunn, P.D., Hussey, B.S., Huang, T., Osterrieder, N., Powell, C., Hand, J., Holz, C., Slater, J., 2013: Experimental infection with equine herpesvirus type 1 (EHV - 1) induces chorioretinal lesions. Vet Res. 44, 118.

Kirisawa, R., Endo, A., Iwai, H., Kawakami, Y., 1993: Detection and identification of equine herpesvirus-1 and - 4 by polymerase chain reaction. Vet Microbiol. 36, 57 - 67.

Kydd, J. H., Slater, J., Osterrieder, N., Lunn, D.P., Antczak, D.F., Azab, W., Balasuriya, U., Barnett, C., Brosnahan, M., Cook, C., Damiani, A., Elton, D., Frampton, A., Gilkerson, J., Goehring, L., Horohov, D., Maxwell, L., Minke, J., Morley, P., Nauwynck, H., Newton, R., Perkins, G., Pusterla, N., Soboll-Hussey, G., Traub-Dargatz, J., Townsend, H., Van de walle, G.R., Wagner, B., 2012: Third International Havemeyer Workshop on Equine Herpesvirus type - 1. Equine Vet J. 44, 513 - 517.

Kydd, J.H, Townsend, H.G., Hannant, D., 2006: The equine immune response to equine herpesvirus - 1: The virus and its vaccines. Vet Immunol Immunopathol. 111, 15 - 30.

Lunn, D.P., Davis-Poynter, N., Flaminio, M.J.B.F., Horohov, D.W., Osterrieder, K., Pusterla, N., Townsend, H.G.G., 2009: EHV-1 Consensus Statement. J Vet Intern Med. 23, 450 - 461.

McCartan, C.G., Russell, M.M., Wood, J.L., Mumford, J.A., 1995: Clinical, serological and virological characteristics of an outbreak of paresis and neonatal foal disease due to equine herpesvirus - 1 on a stud farm. Vet Rec. 136, 7 - 12.

Nugent, J., Birch-Machin, I., Smith, K.C., Mumford, J.A., Swann, Z., Newton, J.R., Bowden, R.J., Allen, G.P., Davis-Poynter, N., 2006: Analysis of equid herpesvirus - 1 strain variation reveals a point mutation of the DNA polymerase strongly associated with neuropathogenic versus non-neuropathogenic disease outbreaks. J Virol. 80, 4047 - 4060.

Perkins, G.A., Goodman, L.B., Tsujimura, K., van de Walle, G.R., Kim, S.G., Dubovi, E.J., Osterrieder, N., 2009: Investigation of the prevalence of neurologic equine herpesvirus type 1 (EHV-1) in a 23-year retrospective analysis (1984 - 2007). Vet Microbiol. 139, 375 - 378.

Pronost, S., Leon, A., Legrand, L., Fortier, C., Miszczak, F., Freymuth, F., Fortier, G., 2010: Neuropathogenic and non-neuropathogenic variants of equine herpesvirus - 1 in France. Vet Microbiol. 145 (3 - 4), 329 - 333.

Pusterla, N., Hussey, S.G., 2014: Equine Herpesvirus - 1 Myeloencephalopathy. Vet Clin North Am Equine Pract. 30 (3), 489 - 506.

Pusterla, N., Mapes, S., Wademan, C., White, A., Estell, K., Swain, E., 2012: Investigation of the role of mules as silent shedders of EHV-1 during an outbreak of EHV - 1 myeloencephalopathy in California. Vet Rec. 170, 465.

Pusterla, N., David, W.W., Madigan, J.E., Ferraro, G.L., 2009: Equine herpesvirus - 1 myeloencephalopathy: a review of recent developments. Vet. J. 180, 279 - 289.

Smith, K.L., Allen, G.P., Branscum, A.J., Frank Cook, R., Vickers, M.L., Timoney, P.J., Balasuriya, U.B., 2010: The increased prevalence of neuropathogenic strains of EHV-1 in equine abortions. Vet Microbiol. 141, 5 - 11.

Telford, E.A., Watson, M.S., Mcbride, K., Davison, A.J., 1992: The DNA Sequence of Equine Herpesvirus - l. Virology, 189, 304 - 316.

USDA-APHIS, 2007: Equine Herpesvirus Myeloencephalopathy: A Potential Emerging Disease. http://www.aphis.usda.gov/animal_health/emergingissues/downloads/ehv1final.pdf. Retrieved on 1 February 2015.

Van de Walle, G.R., Goupil, R., Wishon, C., Damiani, A., Perkins, G.A., Osterrieder, N., 2009: A single-nucleotide polymorphism in a herpesvirus DNA polymerase is sufficient to cause lethal neurological disease. J Infect Dis. 200, 20 - 25.

Van Maanen, C., 2002: Equine herpesvirus - 1 and 4 infection: an update. Vet. Q. 24, 58 – 78.

Van Maanen, C., Sloet van Oldruitenborgh-Oosterbaan, M.M., Damen, E.A., Derksen, A.G., 2001: Neurological disease associated with EHV-1 infection in a riding school: clinical and virological characteristics. Equine Vet J. 33, 191 - 196.

Vandekerckhove, A.P., Glorieux, S., Gryspeerdt, A.C., Steukers, L., Duchateau, L., Osterrieder, N., Van de Walle, G.R., Nauwynck, H.J., 2010: Replication kinetics of neurovirulent versus non-neurovirulent equine herpesvirus type - 1 strains in equine nasal mucosal explants. J Gen Virol. 91, 2019 - 2028.

Vissani, M.A., Becerra, M.L., Olguin Perglione, C., Tordoya, M.S., Mino, S., Barrandeguy, M., 2009: Neuropathogenic and non-neuropathogenic genotypes of equid herpesvirus type - 1 in Argentina. Vet Microbiol. 139, 361 - 364.

Wagner, W.N., Bogdan, J., Haines, D., Townsend, H.G., Misra, V., 1992: Detection of equine herpesvirus and differentiation of equine herpesvirus type - 1 from type 4 by the polymerase chain reaction. Can J Microbiol. 38 (11), 1193 – 119.

CHAPTER 3.2

Detection of equine herpesvirus (EHV) -1, -2, -4, and -5 in Ethiopian equids with and without respiratory problems and genetic characterization of EHV-2 and -5 strains

Adapted from

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Abstract

Infections with equine herpesviruses (EHVs) are widespread in equine populations worldwide. Whereas both EHV-1 and EHV-4 produce well-documented respiratory syndromes in equids, the contribution of EHV-2 and EHV-5 to disease of the respiratory tract is still enigmatic. The present study describes the detection and genetic characterization of EHVs from equids with and without clinical respiratory disease. Virus-specific PCRs were used to detect EHV-1, -2, -4, and -5. From the total of 160 equids with respiratory disease, EHV-5 was detected at the highest prevalence (23.1%), followed by EHV-2 (20.0%), EHV-4 (8.1%), and EHV-1 (7.5%). Concurrent infections with EHV-2 and EHV-5 were recorded from 9 (5.2%) diseased horses. Of the total of 111 clinically healthy equids, EHV-1 and EHV-4 were never detected whereas EHV-2 and EHV-5 were found in 8 (7.2%) and 18 (16.2%) horses, respectively. A significantly higher proportion of EHV-2-infected equids was observed in the respiratory disease group (32/160, 20.0%; P = 0.005) compared to those without disease (8/111; 7.2%). EHV-2-positive equids were three times more likely to display clinical signs of respiratory disease than EHV-2-negative equids (OR 3.22, 95% CI: 1.42 to 7.28). For EHV-5, the observed difference was not statistically significantly (P = 0.166). The phylogenetic analysis of the gB gene revealed that the Ethiopian EHV-2 and EHV-5 strains had a remarkable genetic diversity, with a nucleotide sequence identity among each other that ranged from 94.0 to 99.4% and 95.1 to 100%, respectively. Moreover, the nucleotide sequence identity of EHV-2 and EHV-5 with isolates from other countries acquired from GenBank ranged from 92.9 to 99.1% and 95.1 to 99.5%, respectively. Our results suggest that besides EHV-1 and EHV-4, EHV-2 is likely to be an important contributor either to induce or predispose equids to respiratory disease. However, more work is needed to better understand the contribution of EHV-2 in the establishment of respiratory disease.

Introduction

Equine herpesvirus 1 (EHV-1) and equine herpesvirus 4 (EHV-4) are closely related alphaherpesviruses that cause economically important diseases in equids globally. They are among the most common respiratory tract pathogens in equids (Allen et al., 2004; Gonzalez-Medina & Newton, 2015). EHV-4 mainly infects epithelial cells whereas EHV-1 also infects immune cells, helping the virus to spread rapidly through the basement membrane of the respiratory mucosal epithelial cells and causing a systemic infection, resulting in abortion, myeloencephalopathy, and chorioretinopathy (Allen et al., 2004; Lunn et al., 2009; Hussey et al., 2013).

Equine herpesvirus 2 (EHV-2) and equine herpesvirus 5 (EHV-5) are distinct, but closely related, equid gammaherpesviruses that have been detected in equine populations worldwide (Allen and Murray, 2004). Although the clinical importance is less clearly defined, reports suggest that they are associated with certain disease complaints, such as respiratory disease (Dunowska et al., 2002; Wang et al., 2007; Ataseven et al., 2010), keratoconjunctivitis (Kershaw et al., 2001; Rushton et al., 2016, 2013), and multinodular pulmonary fibrosis (Williams et al., 2007; Dunowska et al., 2014).

The establishment of lifelong latency in a large proportion of infected animals ensures the survival of herpesviruses in equine populations and enables the virus to be shed sporadically throughout the lifetime of the host (Gilkerson et al., 2015; Gonzalez-Medina and Newton, 2015). EHV-1 and EHV-4 enter into a latent state in cells of the lymphoreticular system and neurons in the trigeminal ganglia (Slater et al., 1994). EHV-2 is latent in B-lymphocytes, macrophages, and possibly Langerhans cells (Drummer et al., 1996; Borchers et al., 1997; Allen and Murray, 2004). The latency sites for EHV-5 are unknown (Gilkerson et al., 2015; Goehring, 2015).

Approximately 60% of the world's horse population and over 95% of all donkeys and mules are found in developing countries (Pritchard et al., 2005). Ethiopia possesses approximately half of Africa's equine population. According to the United Nation Food and Agriculture Organization, there are over 7 million donkeys, mules, and horses in Ethiopia (Anonymous,

2010). These working equids have an essential role in the livelihoods of millions of people in Ethiopia. Infectious diseases are an important constraint to the health and productivity of equines. Respiratory viral diseases, especially those caused by EHVs, have been identified as one of the major health threats to equids, which cause significant economic losses (Allen et al., 2004; Lunn et al., 2009). Despite the economic significance of EHVs, studies on the occurrence and the epidemiology of these viruses in Ethiopian working equids are limited. Therefore, the present study was designed to detect and genetically characterize EHVs from equids with and without clinical respiratory disease and to examine the association between respiratory disease and EHV infections.

Materials and methods

Study area and sampling

This study was conducted from September 2015 to March 2016 in selected districts of the northern part of Ethiopia (Angolelana Tera, Abichuna Gnea, and South Achefer) (Figure 1), where large numbers of equids and a high incidence of EHV-1 outbreaks were recorded (CSA, 2014; Negussie et al., 2015). A total of 271 equids at the age ranging from 6 months to 13 years (median: 5.6 years) were included in this study, of which 111 were clinically normal and 160 had clinical signs associated with respiratory disease such as serous to mucopurulent nasal discharge and/or coughing, accompanied with fever (rectal temperature > 38ºC for donkeys and > 38.5ºC for horses). From the total of 160 clinically sick equids, 62 whole blood (donkeys = 24; horses = 38) and 98 nasal swab samples (donkeys = 17; horses = 81) were collected for the detection of EHV-1, -2, -4, and -5. Similarly, from the total of 111 clinically normal equids, 51 whole blood (donkeys = 8; horses = 43) and 60 nasal swab samples (donkey = 2; horses = 58) were collected. All collected samples were immediately placed in a cooler containing ice for transport to the National Animal Health Diagnostic and Investigation Center, Ethiopia. The peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation using Ficoll-Paque PLUS (GE Health Care, Little Chalfont, Buckinghamshire, UK). Samples were kept at -70°C until further processing.

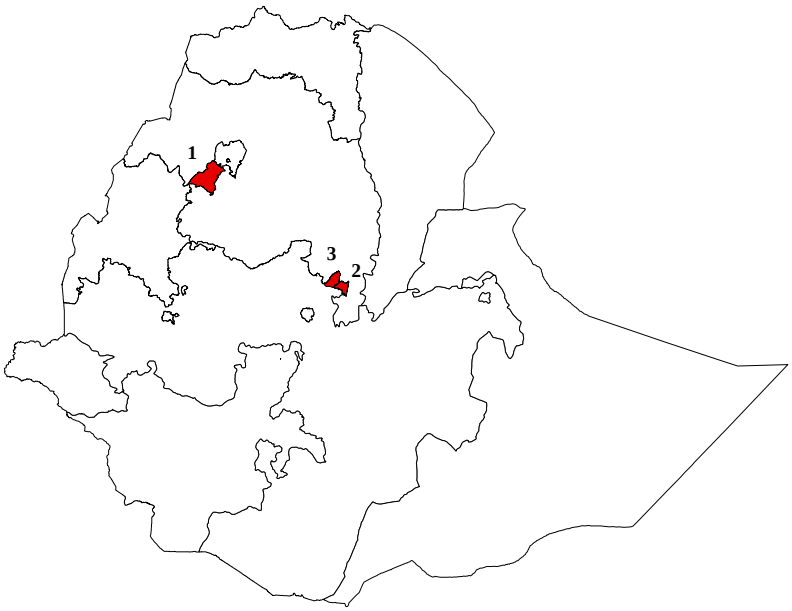

Figure 1: Map of Ethiopia that shows the districts where blood and nasal swab samples were collected from equids with and without respiratory tract disease. 1, South Achefer; 2, AngolelanaTera; 3, Abichuna Gnea

DNA extraction, PCR amplification and sequencing

Viral DNA was extracted from 200µl of PBMCs and nasal swab samples using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. Sensitive virus-specific PCRs targeting the conserved region of glycoprotein B genes were used for each specimen to identify EHV-1, -2, -4, and -5. PCR amplification and subsequent sequencing were performed using virus primers that were specific for the detection and differentiation of closely related viruses such as EHV-1 and EHV-4 (Kirisawa et al., 1993; Léon et al., 2008) and EHV-2 and EHV-5 (Holloway et al., 1999; Dynon et al., 2001; Diallo et al., 2008) (Table 1). PCRs were performed using Agilent's Herculase II fusion DNA polymerase (Agilent Technologies, Inc., Santa Clara, CA, USA). Each of the 25µl PCR mixtures contained 12.5µl of nuclease-free water, 5µl of 5 x Herculase II reaction buffer, 0.5µl Herculase II fusion DNA polymerase, 0.5µl of 25 mM each deoxynucleoside triphosphate (dNTP) mix, 1µl of each forward and reverse primers, 2.5 µl of dimethyl sulphoxide (DMSO), and 2µl template DNA. In each reaction, a positive control (EHV strains that were

isolated and confirmed by sequence analysis) and a negative control (nuclease-free water) were included.

Table 1: Primers used for amplification and sequencing of a specific region of the gB gene of equine herpesviruses

Virus	Region	PCR Primers	Size	Reference
EHV-1	gB	FW: 5'-GCGTTATAGCTATCACGTCC-3' RV: 5'-ATACGATCACATCCAATCCC-3'	190 bp	Kirisawa et al. (1993)
EHV-2	gB	FW: 5'-GCCAGTGTCTGCCAAGTT GATA-3' RV: 5'-CATGGTCTCGATGTCAAACACG-3'	444 bp	Diallo et al. (2008)
EHV-4	gB	FW: 5'-CCTGCATAATGACAGCAGTG-3' RV: 5'-ATACGATCACATCCAATCCC-3'	677 bp	Kirisawa et al. (1993)
EHV-5	gB	FW: 5' ATGAACCTGACAGATGTGCC 3' RV: 5' CACGTTCACTATCACGTCGC 3'	293 bp	Holloway et al. (1999)

The region of interest targeting EHV-1 and EHV-4 gB genes were amplified with an initial denaturation step of 94ºC for 5 min, followed by 40 cycles of denaturation at 94ºC for 30 s, annealing at 64ºC for 30 s, extension at 72ºC for 1 min and 30 s, and a final extension at 72ºC for 10 min. Similarly, EHV-2 and EHV-5 were amplified using the following thermocycling conditions: an initial denaturation step of 95°C for 5 min, followed by 40 cycles of amplification, using denaturation at 95°C for 30 s, annealing at 60ºC, and extension at 72ºC for 45 s and followed by a final extension at 72 °C for 10 min. As a negative control, nuclease-free water was used.

The final specific PCR products were visualized using 1.5% agarose gel electrophoresis. The gels were examined for specific size bands using a UV transilluminator. The PCR products were purified with QIAquick Gel extraction kit (Qiagen) and sequenced by BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on genetic analyzer 3130 (Applied Biosystems) using the same forward and reverse primers as previously described.

Sequence analysis

Sequences were assembled and edited using the DNASTAR software program, SeqMan Pro (version 12; DNASTAR, Inc., Madison, WI, USA). Following sequence assembly, multiple alignments of homologous sequences were analyzed using the ClustalW Multiple alignment programs implemented in BioEdit software version 7. A phylogenetic tree was constructed using the neighbor-joining method in MEGA 6.06 software program. Representative genes of reference strains were retrieved from GenBank. The nucleotide sequences of EHV-2 and EHV-5 strains reported in this studies were submitted to GenBank under the accession number from KX549262 to KX549274.

Statistical analysis

The data were analyzed using SPSS version 20 software (SPSS Inc., Chicago, USA). Pearson Chi-Square test was used to compare the significant difference between the risk factors and EHV infection. The associations between EHV-2 or EHV-5 detection and displaying of clinical signs of the respiratory disease was computed using logistic regression. Differences were considered statistically significant when P-value was < 0.05.

Results

The prevalence of EHV-1, -2, -4, and -5 in equids displaying respiratory disease

Virus-specific PCRs were used to detect EHV-1, EHV-2, EHV-4, and EHV-5 from a total of 160 donkeys and horses exhibiting clinical signs of respiratory disease (nasal discharge and/or coughing, accompanying with fever). EHV-5 was detected at the highest prevalence (37/160; 23.1%), followed by EHV-2 (32/160; 20.0%), EHV-4 (13/160; 8.1%), and EHV-1 (12/160; 7.5%) (Table 2). Concurrent infections with EHV-2 and EHV-5 were recorded from 9 (5.2%) diseased horses.

From the total 62 blood and 98 nasal swab samples collected from equids with signs of respiratory tract disease, EHV-1 was detected from 5 (8.1%) blood and 7 (7.1%) nasal swab samples, EHV-2 was detected from 13 (20.9%) blood and 19 (19.4%) nasal swab samples, EHV-4 was detected from 13 (13.3%) nasal swab samples, and EHV-5 was detected from 19 (30.6%) blood and 18 (18.4%) nasal swab samples.

Both species of equids were infected with EHV-1, with a significantly (P = 0.001) higher prevalence in donkeys (8/41; 19.5%) compared to horses (4/119; 3.4%). Similarly, the prevalence of EHV-4 was somewhat higher in donkeys (4/41; 9.8%) than horses (9/119; 7.6%), however, this difference was not statistically significant (P = 0.658). A significantly (P = 0.005) higher prevalence of EHV-2 and EHV-5 were recorded in horses (EHV-2 (30/119; 25.2%); EHV-5 (34/119; 28.6%)) compared to donkeys (EHV-2 (2/41; 4.9%); EHV-5 (3/41; 7.3%)).

Table 2: The prevalence of EHV-1, -2, -4, and -5 from donkeys and horses displaying clinical signs of respiratory disease

Risk factors	No. of equids	The proportion of equids with respiratory disease that are positive for …..			
		EHV-1	EHV-2	EHV-4	EHV-5
Species					
Horses	119	4 (3.4%)	30 (25.2%)	9 (7.6%)	34 (28.6%)
Donkeys	41	8 (19.5%)	2 (4.9%)	4 (9.8%)	3 (7.3%)
Gender					
Males	48	2 (4.2%)	8 (16.7%)	6 (12.5%)	8 (16.7%)
Females	112	10 (8.9%)	24 (21.4%)	7 (6.2%)	29 (25.9%)
Age					
< 3 years	55	5 (9.1%)	13 (23.6%)	6 (10.9%)	14 (25.5%)
4-6 years	62	4 (6.5%)	11 (17.7%)	6 (9.7%)	15 (24.2%)
7-10 years	34	3 (8.8%)	7 (20.6%)	0 (0.0%)	6 (17.6%)
> 10 years	9	0 (0.0%)	1 (11.1%)	1 (11.1%)	2 (22.2%)
Total no. of equids	160	12 (7.5%)	32 (20.0%)	13 (8.1%)	37 (23.1%)

Although not significantly different, a higher prevalence of EHV-1, EHV-2 and EHV-5 was recorded in females (EHV-1 (10/112; 8.9%; P = 0.295); EHV-2 (24/112; 21.4%; P = 0.490); EHV-5 (29/112; 25.9%; P = 0.205)) than in males (EHV-1 (2/48; 4.2%); EHV-2 (8/48; 16.7%); EHV-5 (8/48; 16.7%)). In contrast, a higher prevalence of EHV-4 was detected in

males (6/48; 12.5%) compared to females (7/112; 6.2%), but the difference was also not statistically significant (P = 0.185).

The age distribution was highly variable ranging from 6 months to 13 years (median: 5.6 years). As shown in Table 2, the animals were classified in the following age groups: less than 3 years (n = 55), 4 to 6 years (n = 62), 7 to 10 years (n = 34), and older than 10 years (n = 9). There was no statistically significant difference among the age groups with EHV infections. However, a higher prevalence of EHV-1 (5/55; 9.1%; P = 0.774), EHV-2 (13/55; 23.6%; P = 0.776), EHV-4 (6/55; 10.9%; P = 0.274), and EHV-5 (14/55; 25.5%; P = 0.853) was recorded at an age less than three years compared to the other age groups.

The prevalence of EHV-1, -2, -4, and -5 in clinically healthy equids

The prevalence of EHV infections was also evaluated from a total of 111 clinically healthy donkeys and horses. EHV-1 and EHV-4 were never detected. EHV-2 and EHV-5 were detected from 8 (7.2%) and 18 (16.2%) horses, respectively. Coinfections with EHV-2 and EHV-5 were not detected from clinically healthy equids (Table 3).

From the total of 51 blood and 60 nasal swab samples collected from clinically healthy equids, EHV-2 was detected from 5 (9.8%) blood and 3 (5.0%) nasal swab samples, whereas EHV-5 was detected from 11 (21.6%) blood and 7 (11.7%) nasal swab samples.

Although not significantly different, the prevalence of EHV-2 was higher in males (3/39; 7.7%; P = 0.884) compared to females (5/72; 6.9%), whereas the prevalence of EHV-5 was higher in females (14/72; 19.4%; P = 0.210) than males (4/39; 10.3%).

A higher prevalence of EHV-2 (3/36; 8.3%; P = 0.986) and EHV-5 (7/36; 19.4%; P = 0.337) was recorded at an age less than three years, but the difference was not statistically significant.

Association between EHV-2 or EHV-5 detection with the presence of respiratory disease

The association of the presence of EHV-2 or EHV-5 with respiratory clinical signs was evaluated by comparing the diseased and healthy group. A significantly higher proportion of EHV-2-infected equids was observed in the respiratory disease group (32/160, 20.0%; P = 0.005) compared to those without disease (8/111; 7.2%). EHV-2-positive equids were three times more likely to display clinical signs of respiratory disease than EHV-2-negative equids (OR 3.22, 95% CI: 1.42 to 7.28). However, a significant association (P = 0.166) was not observed between EHV-5-infected equids in the respiratory disease group (37/160; 23.1%) and those not demonstrating signs of respiratory disease (18/111; 16.2%).

Table 3: The prevalence of EHV-1, -2, -4, and -5 detected from clinically healthy equids

Risk factors	No. of equids	The proportion of equids that are positive for …			
		EHV-1	EHV-2	EHV-4	EHV-5
Species					
Horses	101	0 (0.0%)	8 (7.9%)	0 (0.0%)	18 (17.8%)
Donkeys	10	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Gender					
Males	39	0 (0.0%)	3 (7.7%)	0 (0.0%)	4 (10.3%)
Females	72	0 (0.0%)	5 (6.9%)	0 (0.0%)	14 (19.4%)
Age					
< 3 years	36	0 (0.0%)	3 (8.3%)	0 (0.0%)	7 (19.4%)
4-6 years	33	0 (0.0%)	2 (6.1%)	0 (0.0%)	6 (18.2%)
7-10 years	27	0 (0.0%)	2 (7.4%)	0 (0.0%)	5 (18.5%)
> 10 years	15	0 (0.0%)	1 (6.7%)	0 (0.0%)	0 (0.0%)
Total no. of equids	111	0 (0.0%)	8 (7.2%)	0 (0.0%)	18 (16.2%)

Phylogenetic analysis

The partial nucleotide and amino acid sequences of the gB gene of seven EHV-2 and seven EHV-5 strains obtained from Ethiopian donkeys and horses were compared with each other

and with the sequences of strains from other countries acquired from GenBank (Figure 2). The Ethiopian EHV-2 strains had a genetic diversity, with a nucleotide sequence identity among each other that ranged from 94.0 to 99.4%, and an amino acid sequence identity that ranged from 90.2 to 98.3%. The degree of similarity between Ethiopian strains and foreign EHV-2 strains ranged from 92.9 to 99.1% at nucleotide level and from 87.8 to 98.3% at amino acid level. Similarly, a genetic heterogeneity was observed among Ethiopian EHV-5 strains at nucleotide and amino acid level, with an overall identity of 95.1 to 100% and 94.7 to 100%, respectively. Moreover, EHV-5 showed 95.1 to 99.5% nucleotide and 94.7 to 98.6% amino acid sequence identity with isolates from other countries retrieved from GenBank.

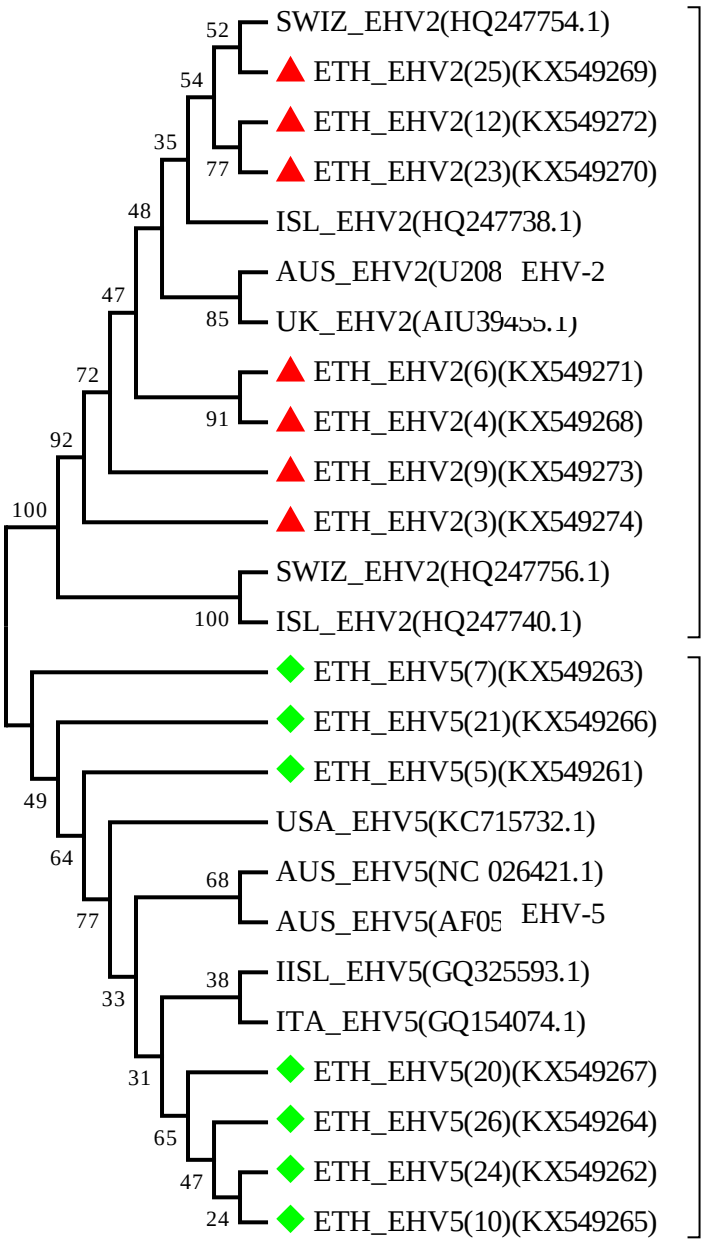

Figure 2: Phylogenetic tree constructed by neighbor-joining method using the gB gene of EHV-2 and EHV-5 from Ethiopian equids. The first two, three or four letters indicate the country of origin followed by strain of the herpesvirus, and then unique identification number. ETH_EHV5(20) (KX549267) and ETH_EHV2(23) (KX549270) are strains from donkeys, while the other strains are from horses. GenBank accession numbers are indicated in between parentheses.

Discussion

Respiratory pathogens are important causes of disease in equine populations worldwide. Although multiple causative agents are involved, viral pathogens play an important role in establishing equine respiratory disease. The EHVs are important viral agents that are involved in equine respiratory problems of varying severity (Allen et al., 2004; Reed and Toribio, 2004). In the present study, the detection and genetic characterization of EHVs from equids with and without respiratory disease are described and the association between respiratory disease and EHV infection is examined. Previously, we described the EHV-1-associated myeloencephalopathy outbreaks in Ethiopian equids (Negussie et al., 2015). In the present study, we also provide the first molecular evidence of EHV-1, EHV-2, EHV-4, and EHV-5 infection in Ethiopian donkeys and horses with and without respiratory problems. A total of 12 (7.5%) and 13 (8.2%) equids exhibiting clinical signs of respiratory disease were infected with EHV-1 and EHV-4, respectively. There is unequivocal evidence that EHV-1 and EHV-4 are major pathogens that cause acute respiratory disease in equids as described elsewhere (Gilkerson et al., 2015). Similarly, 32 (20.0%) and 37 (23.1%) equids with signs of clinical respiratory disease were infected with EHV-2 and EHV-5, respectively. The prevalence of EHV-2 and EHV-5 infections in Ethiopian equids is consistent with a report from Turkey (Ataseven et al., 2010), but relatively lower than other reports from Sweden, Hungary and UK (Back et al., 2009; Nordengrahn et al., 1996) and Australia (Wang et al., 2007). EHV-5 was detected at a higher prevalence than EHV-2, which is in agreement with other reports from Australia (Wang et al., 2007; Diallo et al., 2008) and Turkey (Ataseven et al., 2010), but in contrast to studies from Sweden, Hungary and UK (Nordengrahn et al., 2002), New Zealand (Dunowska et al., 2002) and Iceland (Torfason et al., 2008), where EHV-2 has been more commonly identified. These data indicated that the prevalence of EHV-2 and EHV-5 infections in equine populations are geographically variable, which might be influenced by the breeds of equids and environmental factors.

In this study, EHV-1, -2 and -5 were detetcted from blood and nasal swab samples collected from equids with respiratory disease. EHV-4 was detected only from nasal swab samples. Detection of EHVs in blood samples demonstarting the presence of viremia, probably

resulting from lytic infection, whereas dectection of EHVs in the nasal swab samples suggest viral shedding.

In this study, concurrent infections with EHV-2 and EHV-5 were recorded in 9 (5.2%) horses with respiratory disease. Co-infection was not detected in donkeys. This dual infection is consistent with other reports (Nordengrahn et al., 2002; Back et al., 2009; Ataseven et al., 2010), in which both viruses can simultaneously infect horses. Although we clearly demonstrated that dual infections may be detected in horses exhibiting clinical signs of respiratory disease, their synergistic pathogenic effect on the respiratory tract remains to be determined.

In the current study, both equid species were infected with EHV-1, with a significantly higher prevalence in donkeys compared to horses. This is in line with the previous report of neurological EHV-1 outbreaks in Ethiopia (Negussie et al., 2015), where a larger population of donkeys was affected than horses. The higher prevalence of EHV infections in Ethiopian donkeys might be associated with (1) host factors (Ethiopian donkeys may be more susceptible to EHV infections than horses) and/or (2) stress (donkeys are more subjected to a heavy workload, travel longer distances, are generally in a poor nutritional state, and have a heavy parasite burden). In contrast, a significantly higher prevalence of EHV-2 and EHV-5 were recorded in horses than in donkeys. This is in agreement with previous studies where EHV-2 and EHV-5 have predominantly been found in horses (Franchini et al., 1997; Craig et al., 2005; Goehring, 2015). However, information regarding infection of donkeys with either EHV-2 or EHV-5 is scarce. Borchers et al. (1999) documented that other than horses, the Przewalski's wild horse and the mountain zebras have been identified to be susceptible for EHV-2 and EHV-5. This detection of EHV-2 and EHV-5 in donkeys is the first report, which provides an important contribution to a better understanding of the epidemiology of the disease. At present, a possible explanation why donkeys and horses have a varying susceptibility to EHV infection could not be given.

In the present study, a significant variation was not observed among the age groups with EHV infections. However, the highest prevalence of EHV-1, -2, -4, and -5 was recorded at an age less than three years. This is in line with what has been reported in other studies,

where young equids are at greater risk of developing a clinical respiratory disease associated with EHV infections (Bell et al., 2006; Carlson et al., 2013; Hue et al., 2014; Gilkerson et al., 2015). Young foals are probably infected from their dam by direct contact during the first months, after which the virus is transmitted horizontally to in contact foals (Bell et al., 2006; Brault et al., 2011; Hue et al., 2014).

From the total of 111 clinically healthy equids, EHV-1 and EHV-4 were never detected whereas EHV-2 and EHV-5 were recorded from 8 (7.2%) and 18 (16.2%) horses, respectively. This detection of EHV-2 and EHV-5 from clinically healthy equids is in agreement with previous reports, in which both viruses have been detected from immunocompetent equids without signs of respiratory syndromes (Bell et al., 2006; Wang et al., 2007; Torfason et al., 2008). Our result supports the statement that presence of EHV-2 and/or EHV-5 in equids is not sufficient to establish disease causality (Brault and MacLachlan, 2011). However, they may compromise host immunity and increase the susceptibility of the host to other infections (Nordengrahn et al., 1996; Dunowska et al., 2002). EHV-2 and EHV-5 were not detected from clinically healthy donkeys. This might be due to the small number of donkeys enclosed in this study.

The association of either EHV-2 or EHV-5 detection with the presence of respiratory clinical signs was compared between equids displaying signs of respiratory disease and clinically healthy equids. A significant difference was not observed between EHV-5-infected equids in the respiratory disease group and those not displaying signs of respiratory disease. The high prevalence of EHV-5 in clinically normal equids together with the absence of significant difference between the respiratory diseased and clinically healthy equids may suggest that EHV-5 is unlikely to be involved in clinical respiratory disease in equids. In contrast, a significantly higher proportion of EHV-2-infected equids was observed in the respiratory disease group compared to those without the disease. EHV-2-positive equids were three times more likely to display clinical signs of respiratory disease than EHV-2-negative equids. This strong association proposes that EHV-2 may play a possible etiological contribution either to induce or predispose equids to respiratory diseases. However, further studies are needed to better understand the clinical outcome of EHV-2 and EHV-5 infections.

The partial sequences of the gB gene of EHV-2 and EHV-5 strains were used to compare their phylogenetic relationship with each other and with reference strains from Genbank. The phylogenetic analysis of the gB gene showed that EHV-2 and EHV-5 strains detected in Ethiopia showed a remarkable genetic diversity. EHV-2 had 94 to 99.4% nucleotide sequence identity and 90.2 to 98.3% amino acid sequence identity among each other, whereas the degree of similarity with reference strains ranged from 92.9 to 99.1% and from 87.8 to 98.3% at the level of nucleotide and amino acid sequence, respectively. Similarly, the Ethiopian EHV-5 strains showed an overall identity of 95.1 to 100% and 94.7 to 100% nucleotide and amino acid level, respectively. Moreover, EHV-5 showed 95.1 to 99.5% nucleotide and 94.7 to 98.6% amino acid sequence identity with reference strains from GenBank. This high degree of genetic heterogeneity in equine gammaherpesviruses EHV-2 and EHV-5 is in agreement with previous studies conducted elsewhere (Ataseven et al., 2010; Bell et al., 2006; Brault et al., 2011; Thorsteinsdóttir et al., 2013). Whether this genetic heterogeneity of the gammaherpesviruses has an association with the clinical outcome or not is not clear and deserves further investigation.

In conclusion, both equine alphaherpesviruses (EHV-1 and EHV-4) and gammaherpesviruses (EHV-2 and EHV-5) are common respiratory tract pathogens in donkeys and horses in Ethiopia. EHV-1 and EHV-4 are detected only from equids with respiratory diseases, whereas EHV-2 and EHV-5 are detected from both clinically sick and clinically healthy equids. The partial sequences of the gB gene of Ethiopian EHV-2 and EHV-5 strains have a high degree of genetic heterogeneity. Our results suggest that EHV-2 is likely to be an important contributor either to induce or predispose equids to respiratory diseases. However, more work is needed to better understand the role of EHV-2 in the establishment of respiratory disease.

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References

Allen, G., Murray, M., 2004. Equid herpesvirus 2 and equid herpesvirus 5 infections, in: Infectious Diseases of Livestock, (Ed.) JAW Coetzer and RC Tustin. Oxford Press (Cape Town). pp. 860–867.

Allen, G.P., Kydd, J.H., Slater, J.D., Smith, K.C., 2004. Equid herpesvirus 1 and equid herpesvirus 4 infections., in: JAW Coetzer and RC Tustin (Ed.), Infectious Diseases of Livestock. Oxford Press, Cape Town, South Africa, pp. 829–859.

Anonymous, 2010. FAOSTAT|© FAO Statistics Division. Http://Faostat.Fao.Org/. Accessed April 10, 2016.

Ataseven, V.S., Bilge-Dagalp, S., Oguzoglu, T.C., Karapinar, Z., Guzel, M., Tan, M.T., 2010. Detection and sequence analysis of equine gammaherpesviruses from horses with respiratory tract disease in Turkey. Transbound. Emerg. Dis. 57, 271–276.

Back, H., Ullman, K., Treiberg, L., Riihimäki, M., Penell, J., Ståhl, K., Valarcher, J., Pringle, J., 2009. Viral load of equine herpesviruses 2 and 5 in nasal swabs of actively racing Standardbred trotters: Temporal relationship of shedding to clinical findings and poor performance. Vet. Microbiol. 179, 142–148.

Bell, S.A., Balasuriya, U.B., Nordhausen, R.W., MacLachlan, N.J., 2006. Isolation of equine herpesvirus-5 from blood mononuclear cells of a gelding. J Vet Diagn Invest 18, 472–475.

Bell, S.A., Balasuriya, U.B.R., Gardner, I.A., Barry, P.A., Wilson, W.D., Ferraro, G.L., MacLachlan, N.J., 2006. Temporal detection of equine herpesvirus infections of a cohort of mares and their foals. Vet. Microbiol. 116, 249–257.

Borchers, K., Frölich, K., Ludwig, H., 1999. Detection of equine herpesvirus types 2 and 5 (EHV-2 and EHV-5) in Przewalski's wild horses. Arch. Virol. 144, 771–780.

Borchers, K., Wolfinger, U., Goltz, M., Broll, H., Ludwig, H., 1997. Distribution and relevance of equine herpesvirus type 2 (EHV-2) infections population . Arch. Virol 142, 917–928.

Brault, S.A., Bird, B.H., Balasuriya, U.B.R., MacLachlan, N.J., 2011. Genetic heterogeneity

and variation in viral load during equid herpesvirus-2 infection of foals. Vet. Microbiol. 147, 253–261.

Brault, S.A., MacLachlan, N.J., 2011. Equid gammaherpesviruses: Persistent bystanders or true pathogens? Vet. J. 187, 14–15.

Carlson, J.K., Traub-Dargatz, J.L., Lunn, D.P., Morley, P.S., Kohler, A., Kasper, K., Landolt, G.A., Barnett, D.C., Lunn, K.F., 2013. Equine Viral Respiratory Pathogen Surveillance at Horse Shows and Sales. J. Equine Vet. Sci. 33, 229–237.

Craig, M.I., Barrandeguy, M.E., Fernández, F.M., 2005. Equine herpesvirus 2 (EHV-2) infection in thoroughbred horses in Argentina. BMC Vet. Res. 1, 9.

CSA, 2014. Federal democratic republic of Ethiopia agricultural sample survey 2013/14. Report on livestock and livestock characteristics (private peasant holdings), Statistical Bulletin, volume II, Addis Ababa, Ethiopia.

Diallo, I.S., Hewitson, G.R., De Jong, A., Kelly, M.A., Wright, D.J., Corney, B.G., Rodwell, B.J., 2008. Equine herpesvirus infections in yearlings in South-East Queensland. Arch. Virol. 153, 1643–1649.

Drummer, H., Reubel, G., Studdert, M., 1996. Equine gammaherpesvirus 2 (EHV-2) is latent in B lymphocytes. Arch. Virol 141, 495–504.

Dunowska, M., Hardcastle, M.R., Tonkin, F.B., 2014. Identification of the first New Zealand case of equine multinodular pulmonary fibrosis. N. Z. Vet. J. 62, 226–231.

Dunowska, M., Wilks, C.R., Studdert, M.J., Meers, J., 2002. Equine respiratory viruses in foals in New Zealand. N. Z. Vet. J. 50, 140–147.

Dynon, K., Varrasso, A., Ficorilli, N., Holloway, S.A., Reubel, G.H., Li, F., Hartley, C.A., Studdert, M.J., 2001. Identification of equine herpesvirus 3 (equine coital exanthema virus), equine gammaherpesviruses 2 and 5, equine adenoviruses 1 and 2, equine arteritis virus and equine rhinitis A virus by polymerase chain reaction. Aust. Vet. J. 79, 695–702.

Franchini, M., Akens, M., Bracher, V., von Fellenberg, R., 1997. Characterisation of gamma herpesviruses in the horse by PCR. Virology 238, 8–13.

Gilkerson, J.R., Bailey, K.E., Diaz-Méndez, A., Hartley, C.A., 2015. Update on Viral Diseases of the Equine Respiratory Tract. Vet. Clin. North Am. Equine Pract. 31, 91–104.

Goehring, L.S., 2015. γ-Herpesviruses in Horses and Donkeys, in: Robinson's Current

Therapy in Equine Medicine. Elsevier Inc., pp. 155–157.

Gonzalez-Medina, S., Newton, J.R., 2015. Equine herpesvirus-1: Dealing practically but effectively with an ever present threat. Equine Vet. J. 47, 142–144.

Holloway, S.A., Lindquester, G.J., Studdert, M.J., Drummer, H.E., 1999. Identification, sequence analysis and characterisation of equine herpesvirus 5 glycoprotein B. Arch. Virol. 144, 287–307.

Hue, E.S., Fortier, G.D., Fortier, C.I., Leon, A.M., Richard, E.A., Legrand, L.J., Pronost, S.L., 2014. Detection and quantitation of equid gammaherpesviruses (EHV-2, EHV-5) in nasal swabs using an accredited standardised quantitative PCR method. J. Virol. Methods 198, 18–25.

Hussey, G.S., Goehring, L.S., Lunn, D.P., Hussey, S.B., Huang, T., Osterrieder, N., Powell, C., Hand, J., Holz, C., Slater, J., 2013. Experimental infection with equine herpesvirus type 1 (EHV-1) induces chorioretinal lesions. Vet. Res. 44, 1–15.

Kershaw, O., Von Oppen, T., Glitz, F., Deegen, E., Ludwig, H., Borchers, K., 2001. Detection of equine herpesvirus type 2 (EHV-2) in horses with keratoconjunctivitis. Virus Res. 80, 93–99.

Kirisawa, R., Endo, A., Iwai, H., Kawakami, Y., 1993. Detection and identification of equine herpesvirus-1 and-4 by polymerase chain reaction. Vet. Microbiol. 36, 57–67.

Léon, A., Fortier, G., Fortier, C., Freymuth, F., Tapprest, J., Leclercq, R., Pronost, S., 2008. Detection of equine herpesviruses in aborted foetuses by consensus PCR. Vet. Microbiol. 126, 20–29.

Lunn, D.P., Horohov, D.W., Osterrieder, K., Pusterla, N., 2009. EHV-1 Consensus Statement. J Vet Intern Med 23, 450–461.

Negussie, H., Gizaw, D., Tessema, T.S., Nauwynck, H.J., 2015. Equine Herpesvirus-1 Myeloencephalopathy, an Emerging Threat of Working Equids in Ethiopia. Transbound. Emerg. Dis. doi:10.1111/tbed.12377.

Nordengrahn, A., Merza, M., Ros, C., Lindholmc, A., Palfl, V., Hannant, D., Belak, S., 2002. Prevalence of equine herpesvirus types 2 and 5 in horse populations by using type-specific PCR assays. Vet. Res. 33, 251–259.

Nordengrahn, A., Rusvai, M., Merza, M., Ekstrom, J., Morein, B., Belak, S., 1996. Equine herpesvirus type 2 (EHV-2) as a predisposing factor for Rhodococcus equi pneumonia in foals: Prevention of the bifactorial disease with EHV-2 immunostimulating

complexes. Vet. Microbiol. 51, 55–68.

Pritchard, J., Lindberg, A., Main, D., Whay, H., 2005. Assessment of the welfare of working horses, mules and donkeys, using health and behaviour parameters. Prev Vet Med 69, 265–83.

Reed, S.M., Toribio, R.E., 2004. Equine herpesvirus 1 and 4. Vet. Clin. North Am. - Equine Pract. 20, 631–642.

Rushton, J.O., Kolodziejek, J., Nell, B., Weissenböck, H., Nowotny, N., 2016. Keratoconjunctivitis in a group of Icelandic horses with suspected gammaherpesvirus involvement. Equine Vet. J. 48, 427–9.

Rushton, J.O., Kolodziejek, J., Tichy, A., Nell, B., Nowotny, N., 2013. Detection of equid herpesviruses 2 and 5 in a herd of 266 Lipizzaners in association with ocular findings. Vet. Microbiol. 164, 139–144.

Slater, J.D., Borchers, K., Thackray, A.M., Field, H.J., 1994. The trigeminal ganglion is a location for equine herpesvirus 1 latency and reactivation in the horse. J. Gen. Virol. 75, 2007–2016.

Thorsteinsdóttir, L., Torfason, E.G., Torsteinsdóttir, S., Svansson, V., 2013. Genetic diversity of equine gammaherpesviruses (γ-EHV) and isolation of a syncytium forming EHV-2 strain from a horse in Iceland. Res. Vet. Sci. 94, 170–177.

Torfason, E.G., Thorsteinsdo´ttir, L., Thorsteinsdo´ttir, S.T., Svansson, V.S., 2008. Study of equid herpesviruses 2 and 5 in Iceland with a type-specific polymerase chain reaction. Res. Vet. Sci. 85, 605–611.

Wang, L., Raidal, S.L., Pizzirani, A., Wilcox, G.E., 2007. Detection of respiratory herpesviruses in foals and adult horses determined by nested multiplex PCR. Vet. Microbiol. 121, 18–28.

Williams, K.J., Maes, R., Del Piero, F., Lim, A., Wise, A., Bolin, D., Caswell, J., Jackson, C., Robinson, N., Derksen, F., 2007. Equine multinodular pulmonary fibrosis: A newly recognized herpesvirus-associated fibrotic lung disease. Vet Pathol. 44, 849–62.

CHAPTER 4

REPLICATION CHARACTERISTICS OF EQUINE HERPESVIRUS 1 AND EQUINE HERPESVIRUS 3: COMPARATIVE ANALYSIS USING EX VIVO TISSUE CULTURES

Adapted from

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Abstract

Replication kinetics and invasion characteristics of equine herpesvirus-1 and -3 (EHV-1/-3) in nasal and vaginal mucosae were compared using explants. The explants were cultured during 96h with little change in viability. The tissues were inoculated with EHV-1 03P37 (neuropathogenic), 97P70 (abortigenic) and EHV-3 04P57, collected at 0, 24, 48 and 72h post-inoculation (pi) and stained for viral antigens. Both EHV-1 and EHV-3 replicated in a plaquewise manner. The plaques were already observed at 24h pi, their size increased over time and did not directly cross the basement membrane. However, EHV-1 infected the monocytic cells and hijacked these cells to invade the lamina propria. In contrast, EHV-3 replication was fully restricted to epithelial cells; the virus did not breach the basement membrane via a direct cell-to-cell spread nor used infected monocytic cells. EHV-1-induced plaques were larger in nasal mucosa compared to the vaginal mucosa. The opposite was found for EHV-3-induced plaques. Both EHV-1 strains replicated with comparable kinetics in the nasal mucosa. However, the extent of replication of the abortigenic strain in vaginal mucosa was significantly higher than that of the neuropathogenic strain. Two-to-five-fold lower numbers of EHV-1-infected monocytic cells underneath the basement membrane were found in vaginal mucosa than in nasal mucosa. Our study has shown that (i) EHV-1 has developed in evolution a predisposition for respiratory mucosa and EHV-3 for vaginal mucosa, (ii) abortigenic EHV-1 replicates better in vaginal mucosa than neuropathogenic EHV-1 and (iii) EHV-3 demonstrated a strict epithelial tropism whereas EHV-1 in addition hijacked monocytic cells to invade the lamina propria.

Introduction

Equine herpesvirus 1 (EHV-1) and equine herpesvirus 3 (EHV-3) cause contagious diseases in equids worldwide (Allen and Umphenour, 2004; Allen et al., 2004). EHV-1 is responsible for respiratory disorders, abortion, neonatal foal death, myeloencephalopathy or chorioretinopathy (Allen et al., 2004; Hussey et al., 2013). EHV-3 is the cause of equine coital exanthema (ECE), a genital disease that is transmitted venereally. This disease is characterized by the development of papules, vesicles, pustules and ulcers in the mucosa of the vagina and vestibule of mares and the penis and prepuce of stallions, in the skin of the perineal region of the mares and occasionally on the skin of the lips and mucosa of the upper respiratory tract (Allen and Umphenour, 2004; Barrandeguy et al., 2010b; Kleiboeker and Chapman, 2004). Both EHV-1 and EHV-3 are members of the subfamily *Alphaherpesvirinae* with about 150 kilobases double-stranded DNA genome, consisting of 76 unique open reading frames (Sijmons et al., 2014; Telford et al., 1992). However, antigenically, genetically and pathogenetically, EHV-1 and EHV-3 are significantly different (Allen and Umphenour, 2004).

Latently infected equines are important biological reservoirs for EHV-1 (Slater et al., 1994; Carvalho et al., 2000) and EHV-3 (Allen and Umphenour, 2004; Barrandeguy et al., 2008). The periodic virus reactivation from latency leads to the production of infectious virus that serves as a source of infection (Allen et al., 2004; Barrandeguy et al., 2008). EHV-1 is transmitted to susceptible equids through direct contact with virus-laden respiratory secretions or indirectly with fomites (Allen et al., 2004). Although EHV-3 is primarily transmitted through coitus, contaminated fomites have also been implicated in its spread (Metcalf, 2001).

After initial infection, EHV-1 replicates in mucosal epithelial cells of the upper respiratory tract and causes erosions and viral shedding into the environment (Allen et al., 2004; Kydd et al., 1994a). The virus then invades the underlying lamina propria by infected immune cells (Gryspeerdt et al., 2010; Kydd et al., 1994a, 1994b). Hereafter, EHV-1 disseminates throughout the body using infected mononuclear cells as Trojan horses (Laval et al., 2014). The cell-associated viremia allows the virus to arrive and replicate in the endothelial cells of the target organs, which leads to vasculitis and ischemic thrombosis (Edington et al., 1986). In contrast, EHV-3 replicates in the stratified epithelium of epidermal tissues present at the mucocutaneous

margins and skin (Allen and Umphenour, 2004). Destruction of the epithelium by the lytic virus infection elicits a vigorous, localized inflammatory response that gives rise to the formation of characteristic cutaneous lesions of ECE (Allen and Umphenour, 2004). Infertility and abortion associated with EHV-3 have not been reported (Pascoe, 1981; Van der Meulen et al., 2006). However, the disease has a negative impact in the equine industry as a result of the forced, temporary disruption of the mating activities of affected stallions and mares (Allen and Umphenour, 2004; Barrandeguy and Thiry, 2012).

Different strains of EHV-1 have a different pathological outcome, which is correlated with the variation in the ability to disseminate and establish infection in vascular endothelial cells of the target organs such as the endometrium, the central nervous system and the eye (Patel et al., 1982; Hussey et al., 2013). The respiratory mucosal surface plays a major role in EHV-1 primary replication and transmission (Kydd et al., 1994b). Variation in the structural barriers, microenvironment and the composition of available target cells on mucosal tissues may dramatically influence the efficiency of EHV-1 replication. Previous studies have shown the invasion mechanisms of EHV-1 in the equine respiratory mucosa using nasal explants (Vandekerckhove et al., 2010) and an *in vivo* experiment (Gryspeerdt et al., 2010). It was demonstrated that during infection of epithelial cells, EHV-1 is infecting mucosal monocytes and is hijacking these cells to invade the deeper connective tissues. Despite these studies, little is known about the replication efficiency of EHV-1 strains in the vaginal epithelial mucosa, which could serve as an alternative EHV-1 portal of entry.

EHV-3 is highly host specific. It replicates only in cell lines derived from equids and a laboratory animal model has not been identified for EHV-3 infection (Allen and Umphenour, 2004; Barrandeguy et al., 2012). To date, experimental studies with EHV-3 have solely been done in the natural hosts, equids. To study early events of EHV-3 mucosal invasion, an alternative *in vitro* model would be very valuable. Previously, the replication characteristics of other equine alphaherpesviruses, EHV-1 and EHV-4, have been studied in respiratory mucosa explants (Vandekerckhove et al., 2011, 2010). The replication of EHV-4 was restricted to the epithelial cells, whereas EHV-1 was also infecting mucosal leukocytes. However, the replication kinetics and the invasion characteristics of EHV-3 in mucosae are still not known.

Therefore, this study was designed to compare the replication kinetics and the invasion characteristics of the neuropathogenic and abortigenic strains of EHV-1 and EHV-3 in the nasal and vaginal mucosae using *ex vivo* tissue cultures.

Materials and methods

Tissue collection

The nasal and vaginal tissues were obtained from healthy horses after slaughter in the abattoir. Tissues were taken from three female horses with an estimated age of 5 - 10 years. The absence of recent EHV-1 and EHV-3 infection was demonstrated by the absence of cytopathic effect upon inoculation of rabbit kidney 13 cell and equine dermal cell lines with tissue suspensions (10% W/V). The stage of the reproductive cycle was also evaluated by visual inspection of the ovaries. All the animals were found to be in diestrus.

All the tissues were collected in transport medium containing phosphate buffered saline (PBS) supplemented with 1µg/ml gentamycin (Invitrogen, Paisley, UK), 1 mg/ml streptomycin (Certa, Braine l'Alleud, Belgium), 1 mg/ml kanamycin (Sigma, St. Louis, MO, USA), 1000 U/ml penicillin (Continental Pharma, Puurs, Belgium) and 5 µg/ml amphotericin B (Bristol-Myers Squibb, New York, USA). All collected tissues were placed in a cooler containing ice for transport to the laboratory and were processed immediately after arrival. The mucosae were stripped from the underlying tissues and dissected into explants of approximately 25 mm^2.

Air-liquid culture model

Cultivation of the explants was performed according to the protocol as previously described (Vandekerckhove et al., 2009; Steukers et al., 2011) with some modifications. Briefly, the explants were cultured using fine-meshed gauze, on 6-well tissue culture plate, in an air-liquid interface with epithelium facing upwards. The explants were cultured with serum-free medium consisting of a 1:1 mixture of Roswell Park Memorial Institute medium (RPMI GlutaMAX™) (Invitrogen) and Dulbecco's Modified Eagle's Medium (DMEM GlutaMAX™) (Invitrogen) supplemented with 1µg/ml gentamycin (Invitrogen), 0.1mg/ml streptomycin (Certa, Braine l'Alleud, Belgium) and 100U/ml penicillin (Continental Pharma, Puurs, Belgium). To mimic an

air-liquid interface as in the living animal, the explants were covered with a thin-film of medium and maintained at 37°C in an atmosphere containing 5% CO_2.

Evaluation of tissue viability

Tissue viability was monitored by evaluating ciliary beating of the epithelial cells of the nasal explants using a light microscope and by quantifying the apoptotic cells using *in situ* cell death detection kit (Roche Diagnostics Corporation, Basel, Switzerland). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), preferentially labels DNA strand breaks generated during apoptosis. The test was performed according to the manufacturer's guidelines on cryosections preserved in methocel® (Sigma) at 0, 24, 48, 72 and 96h of cultivation. In both the epithelium and lamina propria, the percentage of TUNEL-positive cells was quantified in five randomly chosen fields of 100 cells each. TUNEL-positive cells were detected and enumerated by fluorescence microscopy (Leica DMRBE, Wild Leitz GmbH, Heidelberg, Germany).

Virus strains used for infections of the explants

Two Belgian EHV-1 strains, representing neuropathogenic and abortigenic variants of EHV-1, were used in this study. These strains were typed by sequencing the DNA polymerase gene of EHV-1 as previously described (Nugent et al., 2006). The abortigenic EHV-1 strain 97P70 which was isolated from an aborted fetus in 1997 and the neuropathogenic strain 03P37 which was isolated from the peripheral blood mononuclear cells of paralytic horses in 2003, were used. EHV-1 virus stocks used for inoculation of the explants were at the 6^{th} passage; 4 passages in equine embryonic lung cells and 2 subsequent passages in rabbit kidney 13 cells.

The EHV-3 strain 04P57 which was isolated from a horse with typical ECE lesions in Belgium in 2004, was used in this experiment. The virus stock used for inoculation was at the second passage in equine embryonic kidney cells. Genetic and pathogenic differences between EHV-3 strains have not been described and therefore, only one EHV-3 strain was included in this study.

Inoculation of the explants

After 24h of culture, explants were inoculated with the strains of EHV-1 and EHV-3 by submerging the tissue in 1ml of inoculum containing $10^{6.5}$ $TCID_{50}$ for 1h at 37ºC and 5% CO_2. After incubation, explants were washed twice with warm medium and transferred back to the original 6-well plates containing gauze and medium. At 0, 24, 48 and 72h post-inoculation (pi), explants were collected, embedded in methylcellulose medium (Methocel® MC, Sigma-Aldrich, St. Louis) and frozen at -70°C.

Immunofluorescence staining and plaque analysis

A double immunofluorescence staining was performed to detect and localize EHV-1 and EHV-3-infected cells at 0, 24, 48 and 72h pi in 100 consecutive 16µm cryosections of the explants. The cryosections were fixed with 100% methanol for 20 min at -20ºC. The basement membrane (BM) of the tissues was stained with monoclonal mouse anti-collagen VII antibodies (Sigma-Aldrich, St. Louis), followed by Texas red-labeled goat anti-mouse antibody (Invitrogen). Biotinylated equine polyclonal anti-EHV-1 IgG antibodies (Van der Meulen et al., 2000) were used to label the EHV-1 viral antigens and biotinylated rabbit polyclonal anti-EHV-3 IgG antibodies to label EHV-3 viral antigens. Next, fluorescein isothiocyanate (FITC) labeled streptavidin (Invitrogen) was added. Mock-inoculated cryosections were stained as negative controls. In each step, cryosections were incubated at 37ºC for 1h and were washed afterward three times with PBS. Hoechst 33342® staining (molecular probes) was performed to visualize the nuclei of the cells. Then, the cryosections were mounted with glycerol containing antifading agent 1, 4-Diazobicyclo-(2, 2, 2-octane (DABCO®). The plaques were visualized using a confocal fluorescence microscope (Leica DMRBE, Wild Leitz GmbH, Heidelberg, Germany). The number of plaques per $8mm^2$ explants and the plaques size was quantified using Leica LAS AF Lite software. The plaques on the borders and edge of the explants were excluded from analysis.

Identification and quantification of single infected cells

To identify and quantify EHV-1 and EHV-3 infected single cells, a double immunofluorescence staining was performed. At each collection time point, 10µm-thick cryosections of tissue explants were fixed in 100% methanol at -20°C for 20min. For each tissue and each time point, 20 cryosections were stained for each cell surface marker separately. Monoclonal antibodies

DH59B (VMRD, USA), UC F6G-3 (California University, Davis, USA) and 1.9/3.2 (VMRD, USA) were used as markers for CD172a cells of the monocyte lineage, CD3 cells (pan T-lymphocytes) and IgM cells (B-lymphocytes), respectively. Then, the cryosections were incubated with Texas Red®-labeled goat anti-mouse IgG antibodies (Invitrogen). In the second step, EHV-1 and EHV-3 viral proteins were stained with biotinylated equine polyclonal anti-EHV-1 IgG antibodies (Van der Meulen et al., 2000) and biotinylated rabbit polyclonal anti-EHV-3 IgG antibodies, respectively, followed by streptavidin-FITC (Invitrogen). Sections of mock-inoculated explants and isotype-matched irrelevant control antibodies were used as negative controls. In each step, cryosections were incubated at 37°C for 1h and washed three times with PBS. The nuclei were counterstained with Hoechst 33342® for 10min. At each time point, the percentage of EHV-1 infected cells that are marker-positive cells were calculated from 20 cryosections of 10µm thick for each specific marker. All the cryosections were analyzed by confocal microscopy (Leica DMRBE, Wild Leitz GmbH, Heidelberg, Germany).

Statistical analysis

The data were analyzed using SPSS version 20 software (SPSS Inc, Chicago, USA). Differences between the strains, tissues and among time points were compared by the analysis of variance (ANOVA) with *post hoc* multiple comparisons. Mann-Whitney test was also used as a non-parametric test. All data are expressed as means with standard deviation (SD) of three independent experiments. Differences were considered statistically significant when P-value was < 0.05.

Results

Tissue viability

The viability of the cells in the nasal and vaginal explants was evaluated using TUNEL staining to detect DNA fragmentation associated with apoptotic cell death at 0, 24, 48, 72 and 96h of cultivation. During the *ex vivo* tissue cultivation, the number of apoptotic cells in the epithelium slightly, but not significantly, increased over time. The percentage of TUNEL-positive cells in the epithelium of the nasal and vaginal mucosae was 1.7±0.2 and 1.9 ± 0.1, respectively, at 96h

of cultivation (Table 1). In the epithelium of the nasal mucosa, the ciliary beating was observed during the whole experiment (up to 96h of cultivation).

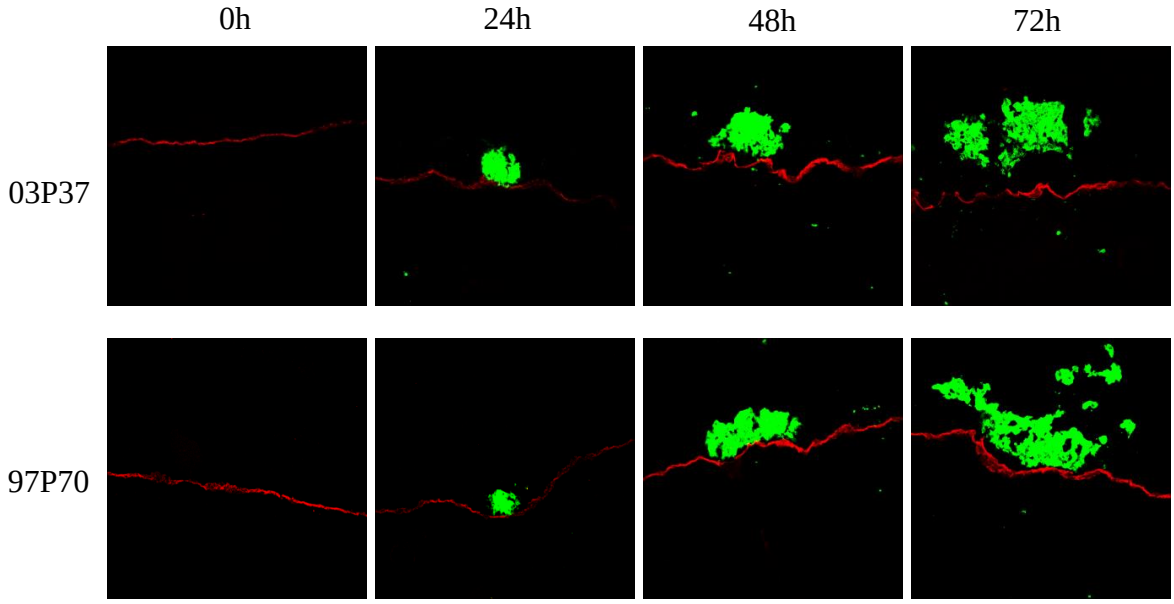
Table 1: Percentage of TUNEL-positive cells in the epithelium and lamina propria of the nasal and vaginal mucosae at different time points of cultivation.

	Percentage of TUNEL-positive cells at indicated time points of cultivation				
	0h	24h	48h	72h	96h
Nasal mucosa					
Epithelium	0.3±0.1	0.4±0.2	0.7±0.1	1.3±0.1	1.7±0.2
Lamina propria	0.7±0.1	1.1±0.3	2.5±0.3	3.1±0.2	4.2±0.2
Vaginal mucosa					
Epithelium	0.4±0.2	0.7±0.1	1.4±0.3	1.7±0.1	1.9±0.1
Lamina propria	0.9±0.2	1.3±0.4	3.4±0.5	3.6±0.6	6.2±0.4

Invasion characteristics

The invasion characteristics of EHV-1 and EHV-3 were assessed in the nasal and vaginal mucosae at 0, 24, 48 and 72h pi. In mock-infected explants, plaques were not observed throughout the experiments. Both strains of EHV-l and EHV-3 replicated in a plaquewise manner and spread laterally in the epithelium. The plaques in the epithelium were already found at 24h pi, their sizes significantly increased over time and they did not cross the BM at all the time points pi. However, EHV-1 and EHV-3 exhibited different invasion characteristics. EHV-1 infects mononuclear immune cells to invade the lamina propria (Figure 1). In contrast, EHV-3 replication was restricted to the epithelium of the nasal and vaginal mucosae, where the virus neither breaches the BM nor infects individual monocytic immune cells at all time points pi (Figure 2).

Nasal mucosa



Vaginal mucosa

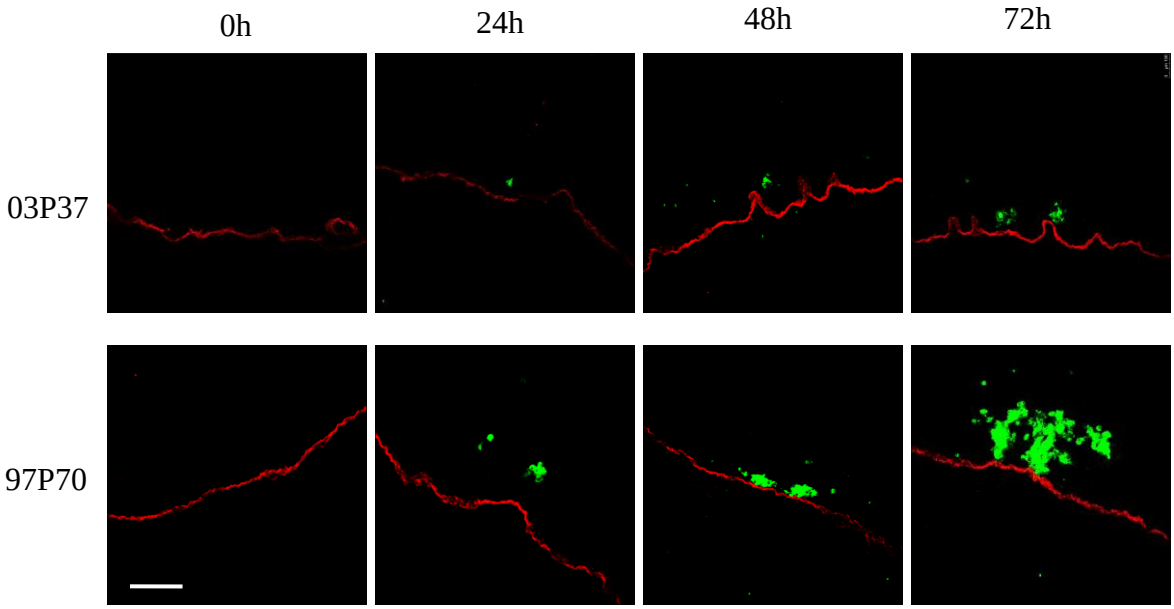


Figure 1: Confocal photomicrographs showing EHV-1-induced plaques in nasal and vaginal explants inoculated with EHV-1 03P37 and EHV-1 97P70. The BM is visualized using mouse anti-collagen VII and goat anti-mouse Texas Red® antibodies. The viral antigens are detected

using biotinylated equine polyclonal anti-EHV-1 IgG antibodies and striptavidin-FITC®. Scale bar: 100µm.

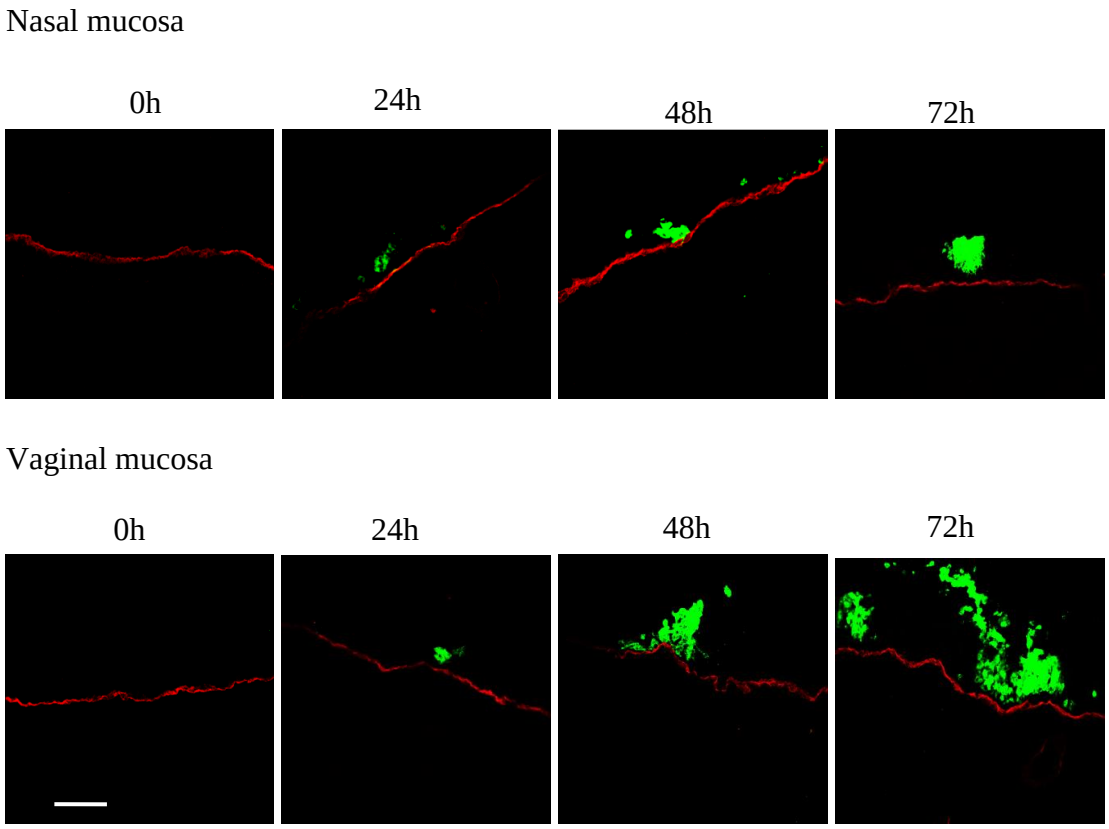


Figure 2: Confocal photomicrographs showing EHV-3-induced plaques in the nasal and vaginal mucosa infected with EHV-3 04P57. The BM is visualized using mouse anti-collagen VII and goat anti-mouse Texas Red® antibodies. The viral antigens are detected using biotinylated rabbit polyclonal anti-EHV-3 IgG antibodies and streptavidin-FITC®. Scale bar: 100µm.

Number of plaques

The number of plaques induced by EHV-1 and EHV-3 on 8mm^2 of explants was counted at 24, 48 and 72h pi. In both mucosae, the average number of plaques induced by both strains of EHV-1 significantly increased ($P < 0.05$) over time. In the nasal mucosa, no significant difference ($P > 0.05$) was observed in the average number of plaques between both EHV-1 strains (Figure 3). However, the average number of plaques in the vaginal mucosa was significantly higher ($P <$

0.05) with the abortigenic strain 97P70 compared to the neuropathogenic strain 03P37 at all time points pi. Overall, the average number of plaques counted in the nasal mucosa was significantly higher ($P < 0.05$) than in the vaginal mucosa.

With EHV-3, the average number of plaques in the nasal mucosa slightly, but not significantly ($P > 0.05$) increased over time. However, the average number of plaques in the vaginal mucosa significantly increased ($P < 0.05$) between 24 and 72h pi. Overall, the average number of plaques was significantly higher ($P < 0.05$) in the vaginal mucosa than in the nasal mucosa at 24 and 72h pi (Figure 3).

Plaques size

Both strains of EHV-1 had a different potential to spread from cell-to-cell in the nasal and vaginal mucosae. In the nasal mucosa**,** the neuropathogenic EHV-1-induced plaques enlarged significantly ($P < 0.05$) over time with an average of 77.9±22.1 µm at 24h pi, 140.1±45.0 µm at 48h pi and 216.1±44.9 µm at 72h pi. Likewise, the size of the plaques induced by the abortigenic strain significantly ($P < 0.05$) increased over time with an average of 68.9±15.8 µm at 24h pi, 166.9±52.3 µm at 48h pi and 245.5±52.3 µm at 72h pi. However, the average sizes of the plaques were not significantly different ($P > 0.05$) between both EHV-1 pathotypes (Figure 3). In the vaginal explants, the size of the plaques induced by the abortigenic strain significantly increased ($P < 0.05$) between 48 and 72h pi with an average of 125±41.7 µm and 192±89.5 µm, respectively. Similarly, the plaque size slightly, but not significantly ($P > 0.05$) increased over time with the neuropathogenic strain. In contrast, the average size of the plaques induced by the neuropathogenic strain was significantly smaller ($P < 0.05$) when compared to the abortigenic strain at all time points pi (Figure 3).

Overall, the average sizes of the plaques induced by both EHV-1 strains were significantly larger ($P < 0.05$) in the nasal mucosa than in the vaginal mucosa.

With EHV-3, the average size of the plaques significantly increased ($p < 0.05$) over time in both the nasal and vaginal explants. The average size of EHV-3-induced plaques in the vaginal mucosa was 56.5±20.1 µm, 84.5±33.8 µm, and 149.2±53.0 µm at 24, 48 and 72h pi, respectively. Similarly, an average of 35.7±12.5 µm (24h pi), 59.6±9.1 µm (48h pi) and

100.8±41.5 µm (72h pi) plaque size was recorded in the nasal mucosa. Overall, the average size of the plaques induced by EHV-3 was significantly higher ($P < 0.05$) in the vaginal mucosa, compared to the nasal mucosa at all time points pi (Figure 3).

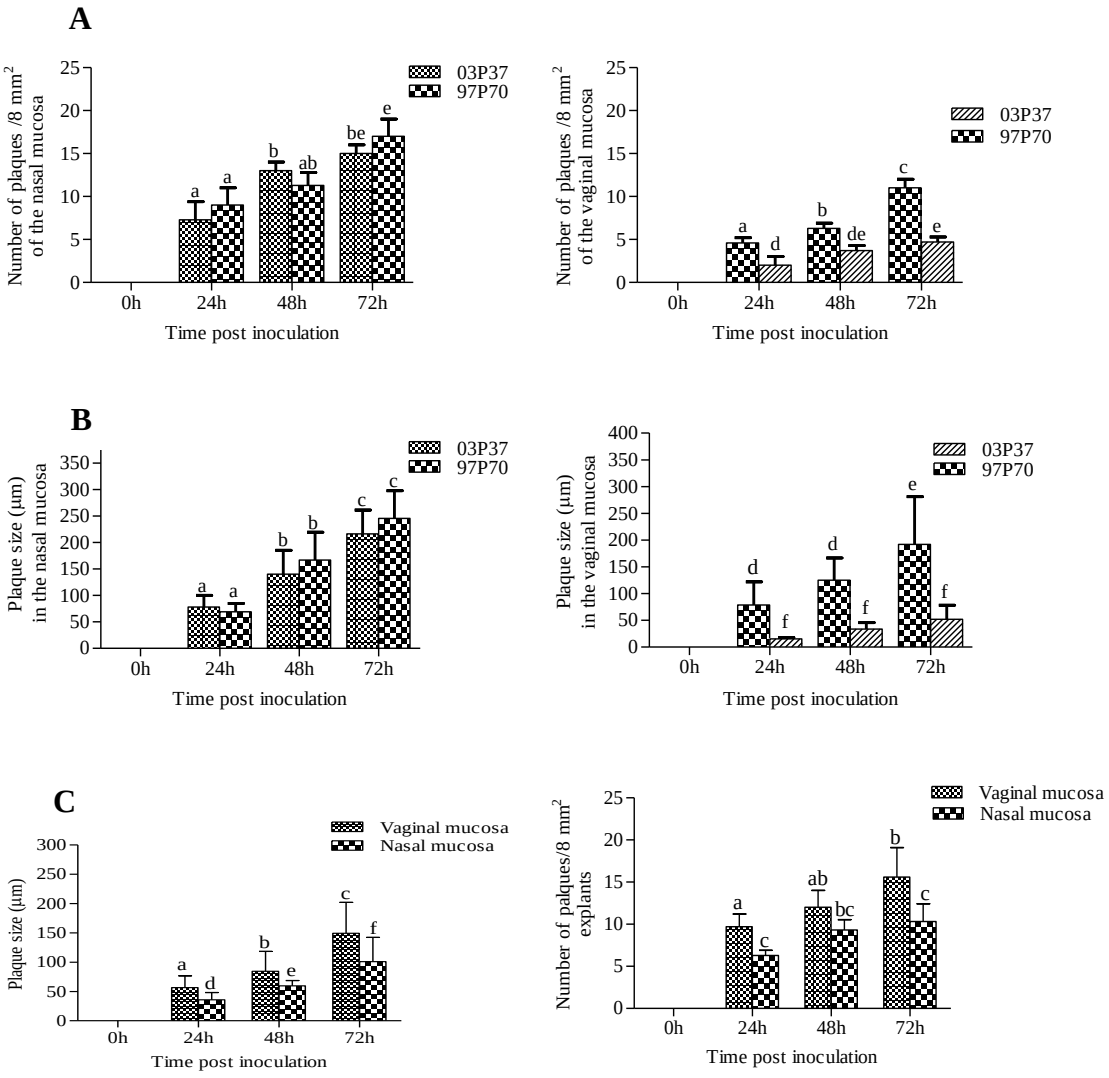


Figure 3: Replication kinetics of EHV-1 (A and B) and EHV-3 (C) in the nasal and vaginal explants. The number of plaques/8mm^2 explants and the plaque size are shown. Data represent means ± SD of triplicate independent experiments. Significant differences are indicated by the use of different letters.

Identification and quantification of single infected cells

Single EHV-1 and EHV-3-infected cells underneath the BM of the nasal and vaginal mucosa tissues were assessed. In both mucosal tissues, EHV-3-infected cells underneath the BM were totally not detected at all the time points pi. In both mucosae, EHV-1-infected cells were already visible starting from 24h pi with the neuropathogenic strain and from 48h with the abortigenic strain (Figure 4).

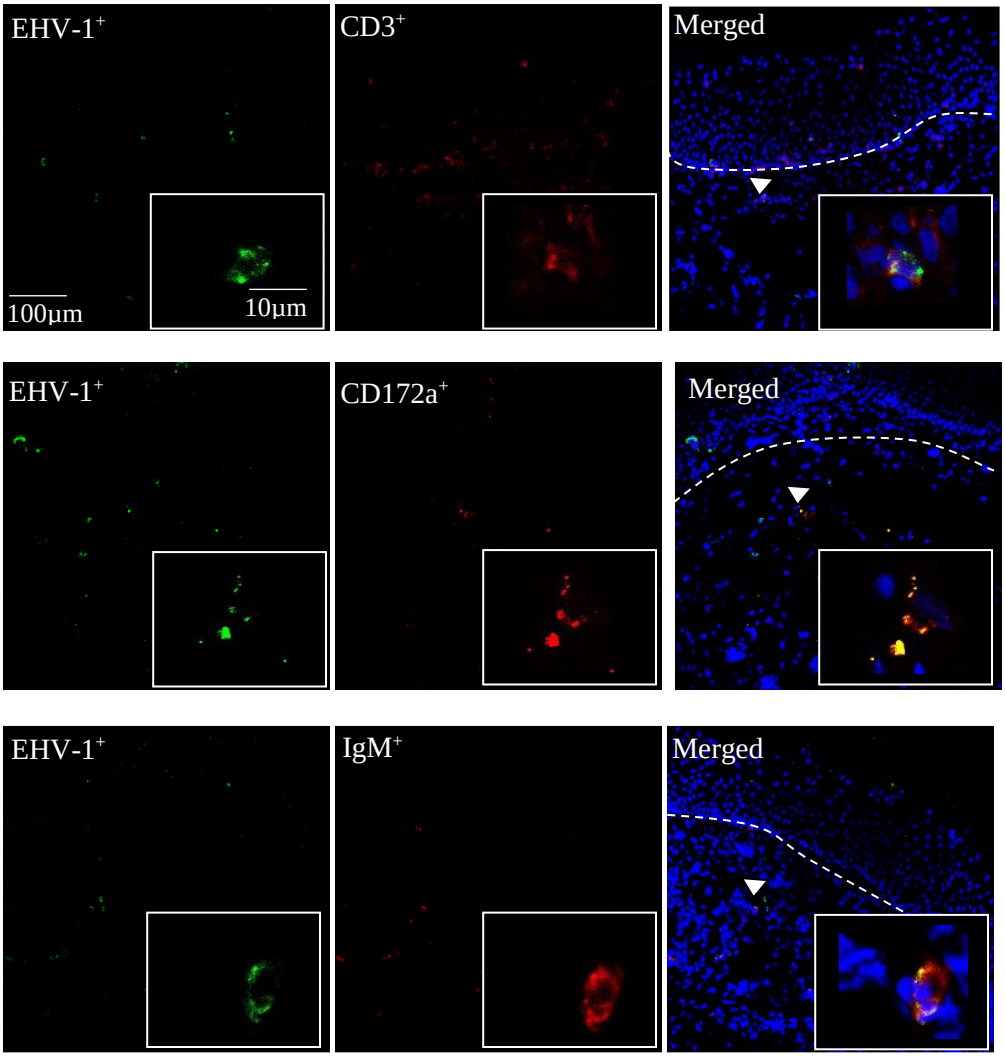
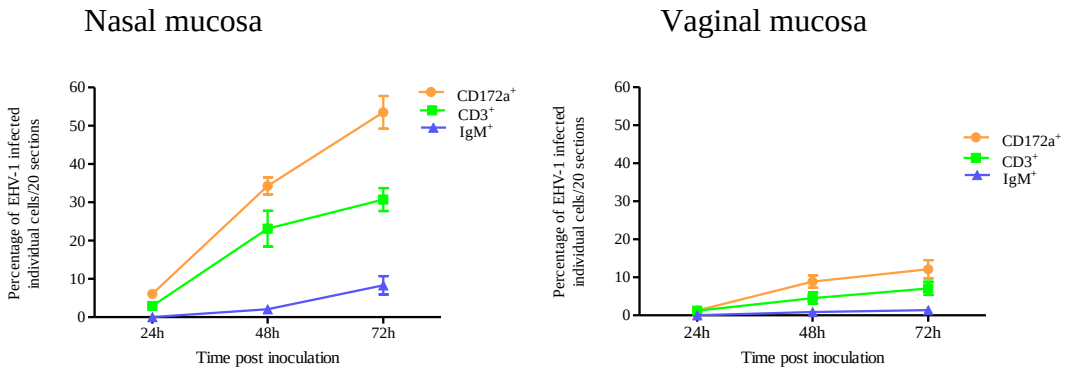


Figure 4: Representative confocal microscope images of marker positive-EHV-1-infected cells in the vaginal mucosa. The explants were sectioned (10µm) and co-immunostained for EHV-1 infected cells (green) and marker-positive cells (red). The white dotted line indicates the BM. White arrowheads show double positive cells.

The percentage of abortigenic EHV-1-infected individual monocytic cells was two-fold lower in the vaginal mucosa than in the nasal mucosa. Similarly, up to a five-fold lower percentage of neuropathogenic EHV-1-infected individual monocytic cells was recorded in the vaginal mucosa than in the nasal mucosa. Regardless of the tissues and strains of EHV-1, $CD172a^+$ cells from the monocytic lineage were the predominant cells type infected, followed by $CD3^+$ T-lymphocytes. EHV-1 infects IgM^+ cells (B-lymphocytes) to a much lesser extent (Figure 5).

A



B

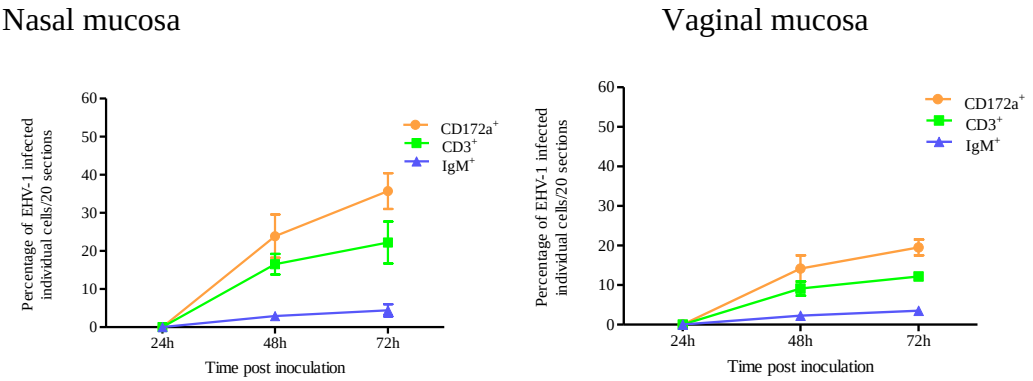


Figure 5: Percentage of EHV-1-infected individual cells. The cells were identified as monocytic cells ($CD172a^+$), pan T-lymphocytes ($CD3^+$) and B-lymphocytes (IgM^+) per 20 sections of the nasal and vaginal mucosal explant infected with EHV-1 03P37 (A) and EHV-1 97P70 (B). Lines show the means ± SD of three independent experiments.

Discussion

Neuropathogenic and abortigenic EHV-1 strains, that display different diseases are circulating in the field. The mucosal surface of the upper respiratory tract plays a major role in EHV-1 replication and transmission. Despite several studies conducted on the replication kinetics and characteristics of EHV-1 on the tissues of the upper respiratory tract, the replication efficiency of both pathotypes in the vaginal mucosa was never studied before. EHV-3 has been associated with both genital and respiratory diseases, however, the underlying pathogenesis remains poorly understood, due in part to the lack of appropriate models to study virus-host interactions. Therefore, in this study, *ex vivo* respiratory and vaginal mucosa explant cultures were developed to gain more insight into the replication kinetics and invasion characteristics of the neuropathogenic and abortigenic strains of EHV-1 and to elucidate the pathogenesis of EHV-3 in the nasal and vaginal mucosae, target organs for virus entry and replication. The explants, directly derived from the upper respiratory tract and the vagina of the horses, were maintained in an air-liquid interface for up to 96h with little change in the viability of the cells as evaluated by ciliary beating and the number of apoptotic cells. The mucosal explant models showed an intact 3-dimensional structure and contained all resident mucosal target cells and consequently are ideal to study the virus-host interactions at the site of infection. These explants from slaughterhouse horses replace experimental animals and, as such, are in line with the 3Rs of animal welfare.

In the present study, both types of EHV-l and EHV-3 replicated in a plaquewise manner and spread laterally on the epithelium. The plaques were already present on the epithelium starting from 24h pi and their sizes significantly increased over time. The plaques did not cross the BM to infect the underlying tissues at all time points pi. However, the invasion via single infected leukocytes in the underlying connective tissue was different. EHV-1 breached the BM and invaded the lamina propria using infected mononuclear immune cells. In contrast, EHV-3 replication was restricted to the epithelial cells, where the virus neither breached the BM in a direct way nor infected individual immune cells to invade the lamina propria. This non-invasive behavior of EHV-3 is comparable with what has been seen for EHV-4 (Vandekerckhove et al., 2011), but is in contrast with other members of alphaherpesviruses such as pseudorabies virus (PRV) (Glorieux et al., 2009), bovine herpesvirus 1 (BoHV-1) (Steukers et al., 2011), herpes

simplex virus 1 (HSV-1) (Glorieux et al., 2011a), canine herpesvirus 1 (CaHV-1) (Li et al., 2016), feline herpesvirus 1 (FeHV-1) (Li et al., 2015) and infectious laryngotracheitis virus (ILTV) (Reddy et al., 2014), which breach the BM and infect the underlying connective tissue. For PRV, it was shown that a cellular serine protease is responsible for this phenomenon (Glorieux et al., 2011b).

In our study, both strains of EHV-1 types have remarkably higher replication kinetics in the nasal mucosa compared to the vaginal mucosa as evaluated by the number of plaques counted, the size of the plaques, and the amount of infected individual mononuclear immune cells. This suggests that the nasal mucosa is the primary tissue of preference for EHV-1 replication and entry into its host. Both EHV-1 types replicated with comparable kinetics in the nasal mucosa at all time points pi. This result is consistent with the earlier report in the respiratory mucosa explants study (Vandekerckhove et al., 2010), where no differences were observed in the replication kinetics between both EHV-1 types. Despite the fact that EHV-1 has a main tropism for the respiratory mucosa, the vaginal mucosa is able to support replication. However, the magnitude of replication in the vaginal mucosa was significantly different between the two EHV-1 strains. The neuropathogenic strain replicated less efficiently than the abortigenic strain and at 72h pi a lot of non-infected basal cells were observed between the cluster of infected epithelial cells and the BM for the neuropathogenic strain but not for the abortigenic strain. Several reasons may be responsible for this observation. Gryspeerdt et al. (2010) reported that after three days of infection the production of interferon limits EHV-1 replication in the epithelium of the upper respiratory tract. Maybe the same defense mechanism is activated in the vaginal mucosa. EHV-1 infection induces high antiviral interferon-α levels, which is critical in the host innate immune response (Wagner et al., 2011). Another possible explanation for the difference in replication efficiency observed between the two EHV-1 strains might be associated with viral genetic factors. Goodman et al., (2007) reported that EHV-1 virulence and tissue tropism in the natural host are linked with the function of the DNA polymerase. Indeed, a single point mutation in this enzyme has been claimed to be responsible for the neurotropism. It would now be very interesting to determine if the same point mutation in this enzyme is also determining the level of EHV-1 replication in the vaginal mucosa. It is very well possible that this point mutation is generally affecting the replication of EHV-1 in the different mucosae of the genital tracts (endometrium and vaginal mucosa).

In this study, although we clearly demonstrated that both EHV-1 types are capable of replicating in the mucosa of the vaginal epithelium, the extent of replication is much higher with the abortigenic strain compared with the neuropathogenic strain. Previous reports indicated that EHV-1 has been isolated from male genital organs (Tearle et al., 1996) and is shed with the semen and the sperm cells (Fritsche and Borchers, 2011; Hebia-Fellah et al., 2009; Walter et al., 2012). When we combine the latter reports with our findings, we could propose a possible venereal transmission of EHV-1 via the semen, which was largely ignored before. The venereal transmission has been well documented for other alphaherpesviruses such as EHV-3 (Allen and Umphenour, 2004), BoHV-1 (Wrathall et al., 2006; Bielanski et al., 2014), PRV (Romero et al., 2001) and CaHV-1 (Evermann et al., 2011). Therefore, our study highlights an important insight that should be further investigated in the field.

In our study, although both the nasal and vaginal mucosae were infected by EHV-3, the replication kinetics are significantly different. The sizes of EHV-3-induced plaques are significantly larger in the vaginal mucosa than the nasal mucosa. This replication advantage of EHV-3 in the vaginal mucosa might be associated with the virus tissue tropism, as it has a higher affinity to affect the genital organs under natural conditions. Although, post-coital infection of the EHV-3 is the general route of transmission, non-venereal transmission to the nasal mucosa has been reported via genitonasal contact and contaminated objects (Allen and Umphenour, 2004; Barrandeguy et al., 2010a). In the current study, EHV-3 replication was restricted to the epithelium mucosae, where the virus neither breached the BM to invade the lamina propria nor infected individual mononuclear immune cells. This localized replication behavior might limit the EHV-3 dissemination via systemic blood circulation. *In vivo* EHV-3 infection destroys the epithelium and elicits a vigorous, localized inflammatory response (Allen and Umphenour, 2004) and systemic dissemination of the virus is exceptional (Barrandeguy et al., 2010a). Whether host factors or viral factors inhibit the virus to infect individual immune cells and to invade the underlying tissue is not clear and deserves further investigation.

In the current study, the neuropathogenic strain infects a higher percentage of monocytic cells in the nasal mucosa when compared to the abortigenic strain. This result is in line with the previous report made by Vandekerckhove et al. (2010) in the nasal explants study and Gryspeerdt et al. (2010) in an *in vivo* study. However, the neuropathogenic strain infects a lower

percentage of cells in the vaginal mucosa than the abortigenic strain of EHV-1. These results suggest that the number of single infected cells may vary with the mucosa type infected with EHV-1. With both EHV-1 types, two-to-five-fold lower percentage of infected monocytic cells were found in the lamina propria of the vaginal mucosa than in that of the nasal mucosa. Infected individual cells underneath the BM were visible already at 24h pi with the neuropathogenic strain, and at 48h pi with the abortigenic strain. The monocytic lineage cells, which express surface marker CD172a, were the predominant cell type infected with EHV-1, independent of the strain and tissue. This is in agreement with the previous report in the nasal explants (Vandekerckhove et al., 2010), in an *in vivo* experiment (Gryspeerdt et al., 2010), and in peripheral blood mononuclear cells (Baghi and Nauwynck, 2014; Laval et al., 2014). This cell marker is expressed in equine monocytes, macrophages, dendritic cells and granulocytes (Van Beek et al., 2005; Barclay and Brown, 2006). Recently, Baghi et al. (2014) reported that isolated equine nasal mucosal $CD172a^+$ cells resemble immature dendritic cells. The dendritic cells in the periphery capture and process antigens, express lymphocyte co-stimulatory molecules, migrate to lymphoid organs and secrete cytokines to initiate immune responses (Banchereau and Steinman, 1998). $CD3^+$ T- lymphocytes were also an important cell type infected with EHV-1. IgM^+ cells (B- lymphocytes) were infected to a much lesser extent.

In conclusion, EHV-1 and EHV-3 exhibited different invasion characteristics. Both viruses replicated in a plaquewise manner and spread laterally on the epithelium. EHV-3-induced plaques are restricted to the epithelium of the nasal and vaginal mucosae and the virus neither breaches the BM nor infect individual immune cells at all time points pi. In contrast, EHV-1 invades the underlying connective tissue by infecting mononuclear immune cells. Our *ex vivo* explant models provided an important new insight that should be further investigated in order to better understand the underlying mechanisms and *in vivo* relevance.

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References

Allen, G., Umphenour, N., 2004. Equine Coital Exanthema, in: Tustin, J.C. and R. (Ed.), Infectious Diseases of Livestock. Oxford Press, Cape Town, South Africa, pp. 860–867.

Allen, G.P., Kydd, J.H., Slater, J.D., Smith, K.C., 2004. Equid herpesvirus 1 and equid herpesvirus 4 infections., in: JAW Coetzer and RC Tustin (Ed.), Infectious Diseases of Livestock. Oxford Press, Cape Town, South Africa, pp. 829–859.

Baghi, H.B., Laval, K., Favoreel, H., Nauwynck, H.J., 2014. Isolation and characterization of equine nasal mucosal CD172a+ cells. Vet. Immunol. Immunopathol. 157, 155–163.

Baghi, H.B., Nauwynck, H.J., 2014. Impact of equine herpesvirus type 1 (EHV-1) infection on the migration of monocytic cells through equine nasal mucosa. Comp. Immunol. Microbiol. Infect. Dis. 37, 321–329.

Banchereau, J., Steinman, R.M., 1998. Dendritic cells and the control of immunity. Nature 392, 245–252.

Barclay, A.N., Brown, M.H., 2006. The SIRP family of receptors and immune regulation. Nat. Rev. Immunol. 6, 457–464.

Barrandeguy, M., Perkins, J., Donough, J.M., Vissani, A., Olguin, C., Thiry, E., 2010a. Occurrence of Equine Coital Exanthema in Mares from an Embryo Transfer Center. J. Equine Vet. Sci. 30, 145–149.

Barrandeguy, M., Thiry, E., 2012. Equine coital exanthema and its potential economic implications for the equine industry. Vet. J. 191, 35–40.

Barrandeguy, M., Ulloa, N., Bok, K., Fernández, F., 2010b. Outbreak of rhinitis caused by equine herpesvirus type 3. Vet. Rec. 166, 178–179.

Barrandeguy, M., Vissani, A., Olguin, C., Barbara, G., Valenzuela, H., Becerra, L., Tordoya, M., Miño, S., Thiry, E., 2012. Experimental infection with equid herpesvirus 3 in seronegative and seropositive mares. Vet. Microbiol. 160, 319–326.

Barrandeguy, M., Vissani, A., Olguin, C., Becerra, L., Miño, S., Pereda, A., Oriol, J., Thiry, E., 2008. Experimental reactivation of equine herpesvirus-3 following corticosteroid treatment. Equine Vet. J. 40, 593–595.

Bielanski, A., Algire, J., Lalonde, A., Garceac, A., 2014. Risk of Transmission of Bovine Herpesvirus-1 (BHV-1) by Infected Semen to Embryo Recipients and Offspring. Reprod. Domest. Anim. 49, 197–201.

Carvalho, R., Oliveira, A.M., Souza, A.M., Passos, L.M., Martins, A.S., 2000. Prevalence of equine herpesvirus type 1 latency detected by polymerase chain reaction. Arch. Virol. 145, 1773–1787.

Edington, N., Bridges, C.G., Patel, J.R., 1986. Endothelial cell infection and thrombosis in paralysis caused by equid herpesvirus-1: Equine stroke. Arch. Virol. 90, 111–124.

Evermann, J.F., Ledbetter, E.C., Maes, R.K., 2011. Canine Reproductive, Respiratory, and Ocular Diseases due to Canine Herpesvirus. Vet. Clin. North Am. - Small Anim. Pract. 41, 1097–1120.

Fritsche, A.K., Borchers, K., 2011. Detection of neuropathogenic strains of Equid Herpesvirus 1 (EHV-1) associated with abortions in Germany. Vet. Microbiol. 147, 176–180.

Glorieux, S., Bachert, C., Favoreel, H.W., Vandekerckhove, A.P., Steukers, L., Rekecki, A., van den Broeck, W., Goossens, J., Croubels, S., Clayton, R.F., Nauwynck, H.J., 2011a. Herpes simplex virus type 1 penetrates the basement membrane in human nasal respiratory mucosa. PLoS One 6, 10–13.

Glorieux, S., Favoreel, H.W., Meesen, G., De Vos, W., Van den Broeck, W., Nauwynck, H.J., 2009. Different replication characteristics of historical pseudorabies virus strains in porcine respiratory nasal mucosa explants. Vet. Microbiol. 136, 341–346.

Glorieux, S., Favoreel, H.W., Steukers, L., Vandekerckhove, A.P., Nauwynck, H.J., 2011b. A trypsin-like serine protease is involved in pseudorabies virus invasion through the basement membrane barrier of porcine nasal respiratory mucosa. Vet. Res. 42, 58.

Goodman, L.B., Loregian, A., Perkins, G.A., Nugent, J., Buckles, E.L., Mercorelli, B., Kydd, J.H., Palù, G., Smith, K.C., Osterrieder, N., Davis-Poynter, N., 2007. A point mutation in a herpesvirus polymerase determines neuropathogenicity. PLoS Pathog. 3, 1583–1592.

Gryspeerdt, A.C., Vandekerckhove, A.P., Garré, B., Barbé, F., Van de Walle, G.R., Nauwynck, H.J., 2010. Differences in replication kinetics and cell tropism between neurovirulent and non-neurovirulent EHV1 strains during the acute phase of infection in horses. Vet.

Microbiol. 142, 242–253.

Hebia-Fellah, I., Léauté, A., Fiéni, F., Zientara, S., Imbert-Marcille, B.M., Besse, B., Fortier, G., Pronost, S., Miszczak, F., Ferry, B., Thorin, C., Pellerin, J.L., Bruyas, J.F., 2009. Evaluation of the presence of equine viral herpesvirus 1 (EHV-1) and equine viral herpesvirus 4 (EHV-4) DNA in stallion semen using polymerase chain reaction (PCR). Theriogenology 71, 1381–1389.

Hussey, G.S., Goehring, L.S., Lunn, D.P., Hussey, S.B., Huang, T., Osterrieder, N., Powell, C., Hand, J., Holz, C., Slater, J., 2013. Experimental infection with equine herpesvirus type 1 (EHV-1) induces chorioretinal lesions. Vet. Res. 44, 1–15.

Kleiboeker, S.B., Chapman, R.K., 2004. Detection of equine herpesvirus 3 in equine skin lesions by polymerase chain reaction. J. Vet. Diagn. Invest. 16, 74–79.

Kydd, J.H., Smith, K.C., Hannant, D., Livesay, G.J., Mumford, J.A., 1994a. Distribution of equid herpesvirus-1 (EHV-1) in respiratory tract associated lymphoid tissue: implications for cellular immunity. Equine Vet. J. 26, 470–473.

Kydd, J.H., Smith, K.C., Hannant, D., Livesay, G.J., Mumford, J.A., 1994b. Distribution of equid herpesvirus-1 (EHV-1) in the respiratory tract of ponies: implications for vaccination strategies. Equine Vet. J. 26, 466–469.

Laval, K., Favoreel, H.W., Nauwynck, H.J., 2014. Equine herpesvirus type 1 replication is delayed in CD172a+ monocytic cells and controlled by histone deacetylases. J. Gen. Virol. 96, 118–130.

Li, Y., Negussie, H., Qiu, Y., Reddy, V.R.A.P., Mateusen, B., Nauwynck, H.J., 2016. Early events of canine herpesvirus 1 infections in canine respiratory and genital mucosae by the use of ex vivo models. Res. Vet. Sci. 105, 205–208.

Li, Y., Van Cleemput, J., Qiu, Y., Reddy, V.R. a. P., Mateusen, B., Nauwynck, H.J., 2015. Ex vivo modeling of feline herpesvirus replication in ocular and respiratory mucosae, the primary targets of infection. Virus Res. 210, 227–231.

Metcalf, E.S., 2001. The role of international transport of equine semen on disease transmission. Anim. Reprod. Sci. 68, 229–237.

Nugent, J., Birch-Machin, I., Smith, K.C., Mumford, J.A., Swann, Z., Newton, J.R., Bowden, R.J., Allen, G.P., Davis-Poynter, N., 2006. Analysis of equid herpesvirus 1 strain variation reveals a point mutation of the DNA polymerase strongly associated with neuropathogenic versus nonneuropathogenic disease outbreaks. J. Virol. 80, 4047–4060.

Pascoe, R.R., 1981. The effect of equine coital exanthema on the fertility of mares covered by stallions exhibiting the clinical disease. Aust. Vet. J. 57, 111–114.

Patel, J.R., Edington, N., Mumford, J.A., 1982. Variation in cellular tropism between isolates of Equine herpesvirus-1 in Foals. Arch. Virol. 74, 41–51.

Reddy, V.R.A.P., Steukers, L., Li, Y., Fuchs, W., Vanderplasschen, A., Nauwynck, H.J., 2014. Replication characteristics of infectious laryngotracheitis virus in the respiratory and conjunctival mucosa. Avian Pathol. 43, 450–457.

Romero, C.H., Meade, P.N., Shultz, J.E., Chung, H.Y., Gibbs, E.P., Hahn, E.C., Lollis, G., 2001. Venereal transmission of pseudorabies viruses indigenous to feral swine. J. Wildl. Dis. 37, 289–296.

Sijmons, S., Vissani, A., Tordoya, S., Muylkens, B., Thiry, E., Maes, P., Matthijnssens, J., 2014. Complete Genome Sequence of Equid Herpesvirus 3. Genome Announc 2, 12–13.

Slater, J.D., Borchers, K., Thackray, A.M., Field, H.J., 1994. The trigeminal ganglion is a location for equine herpesvirus 1 latency and reactivation in the horse. J. Gen. Virol. 75, 2007–2016.

Steukers, L., Vandekerckhove, A.P., Van Den Broeck, W., Glorieux, S., Nauwynck, H.J., 2011. Comparative analysis of replication characteristics of BoHV-1 subtypes in bovine respiratory and genital mucosa explants: A phylogenetic enlightenment. Vet. Res. 42, 33.

Tearle, J.P., Smith, K.C., Boyle, M.S., Binns, M.M., Livesay, G.J., Mumford, J.A., 1996. Replication of equid herpesvirus-1 (EHV-1) in the testes and epididymides of ponies and venereal shedding of infectious virus. J. Comp. Pathol. 115, 385–397.

Telford, E.A.R., Watson, M.S., McBride, K., Davison, A.J., 1992. The DNA sequence of equine herpesvirus-1. Virology 189, 304–316.

Van Beek, E.M., Cochrane, F., Barclay, A.N., Van den Berg, T.K., 2005. Signal regulatory proteins in the immune system. J. Immunol. 175, 7781–7787.

Van der Meulen, K., Caij, A., Smets, K., Nauwynck, H., 2006. Equine coital exanthema in mare in Belgium. Vlaams Diergeneeskd. Tijdschr. 75, 286–289.

Van der Meulen, K.M., Nauwynck, H.J., Buddaert, W., Pensaert, M.B., 2000. Replication of equine herpesvirus type 1 in freshly isolated equine peripheral blood mononuclear cells and changes in susceptibility following mitogen stimulation. J. Gen. Virol. 81, 21–25.

Vandekerckhove, A., Glorieux, S., Broeck, W. Van Den, Gryspeerdt, A., van der Meulen, K.M., Nauwynck, H.J., 2009. In vitro culture of equine respiratory mucosa explants. Vet. J. 181,

280–287.

Vandekerckhove, A.P., Glorieux, S., Gryspeerdt, A.C., Steukers, L., Duchateau, L., Osterrieder, N., Van De Walle, G.R., Nauwynck, H.J., 2010. Replication kinetics of neurovirulent versus non-neurovirulent equine herpesvirus type 1 strains in equine nasal mucosal explants. J. Gen. Virol. 91, 2019–2028.

Vandekerckhove, A.P., Glorieux, S., Gryspeerdt, A.C., Steukers, L., Van Doorsselaere, J., Osterrieder, N., Van de Walle, G.R., Nauwynck, H.J., 2011. Equine alphaherpesviruses (EHV-1 and EHV-4) differ in their efficiency to infect mononuclear cells during early steps of infection in nasal mucosal explants. Vet. Microbiol. 152, 21–28.

Wagner, B., Wimer, C., Freer, H., Osterrieder, N., Erb, H.N., 2011. Infection of peripheral blood mononuclear cells with neuropathogenic equine herpesvirus type-1 strain Ab4 reveals intact interferon-?? induction and induces suppression of anti-inflammatory interleukin-10 responses in comparison to other viral strains. Vet. Immunol. Immunopathol. 143, 116–124.

Walter, J., Balzer, H.J., Seeh, C., Fey, K., Bleul, U., Osterrieder, N., 2012. Venereal Shedding of Equid Herpesvirus-1 (EHV-1) in Naturally Infected Stallions. J. Vet. Intern. Med. 26, 1500–1504.

Wrathall, A.E., Simmons, H.A., Van Soom, A., 2006. Evaluation of risks of viral transmission to recipients of bovine embryos arising from fertilisation with virus-infected semen. Theriogenology 65, 247–274.

CHAPTER 5

GENERAL DISCUSSION

EHV-1 is an important pathogen, causing an array of disorders including respiratory disease in young horses, abortion in pregnant mares, neonatal foal death, myeloencephalopathy, or chorioretinopathy (Allen et al., 2004; Hussey et al., 2013). The main relevant epidemiological feature of EHV-1 is a high incidence of respiratory infection early in life, the establishment of a life-long latent infection in a high percentage of equids, and periodic reactivation of latent virus with subsequent shedding, resulting in horizontal transmission to naive hosts (Allen et al., 2004). Currently, available vaccines do not reliably prevent infection, development of viremia or establishment of latency, and EHM has been reported in horses regularly vaccinated against EHV-1 within 3-5 month intervals (Friday et al., 2000; Henninger et al., 2007). Thus, early recognition of the disease is very crucial for the implementation of management practices that decrease the risk of exposure of susceptible equids. Several epidemiological studies have been conducted on equine herpesviruses, however, many data gaps exist and more investigation needs to be done to better understand the epidemiology of the disease in the different geographical settings and equine populations. Epidemiological investigations of EHV outbreaks, particularly in unvaccinated populations, are very important to improve our understanding of herpesvirus pathogenesis. The analysis of this epidemiological data is also very crucial in order to design more effective intervention strategies. Therefore, in **chapter 3** of this thesis, the epidemiology of EHVs in Ethiopian equids was addressed.

Equine herpesvirus 1 myeloencephalopathy outbreaks in Ethiopian equids

In the first study **(chapter 3.1)** of this thesis, we described the occurrence of EHM outbreaks in horses, mules, and donkeys in Ethiopia. During the period of 2011 to 2013, we recorded 14 EHM outbreaks in 10 districts of the country. We described EHV-1-associated outbreaks that occurred for the first time in Ethiopian equids. We found a large number of clinically affected donkeys, besides horses and mules and an increasing frequency of EHM-associated outbreaks over time. Moreover, we observed an EHM spread at an alarming rate to different parts of Ethiopia. Based on this knowledge, EHM is considered as an emerging threat to Ethiopian equids. EHM has been described for many years, but in recent years, there has been an apparent increase in frequency and severity in most parts of the world. In 2007, the US Department of Agriculture, the Center for Emerging Issues released an emerging disease notice report regarding the neurologic form of EHV-1 (USDA-APHIS, 2007).

In this thesis, we described the clinical signs associated with EHM infection in Ethiopian equids and visible differences in the clinical presentation of EHM among horses, mules, and donkeys were not observed. However, we found large numbers and more severe EHM-associated clinical signs in donkeys within the population. We noticed that donkeys were frequently found dead associated with EHM after showing ataxia and paresis, but without demonstrating clinical signs of complete paralysis and recumbency. In previous studies, donkeys and mules were considered as a silent reservoir host of EHV-1 infections (Van Maanen, 2002; Pusterla et al., 2012). This clinical outcome of EHV-1 infections in Ethiopian equids might be associated with (1) the host factor, where the Ethiopian donkeys might be highly susceptible to EHM infection, (2) the stress factors, where equids are subjected to heavy workload, traveling long distances, poor nutritional state, heavy parasite burden, and concurrent diseases, or (3) a difference in the pathogenic potential of EHV-1 strains. Barbic et al. (2012) and Goehring (2015a) observed a significant influence of breed on the clinical outcome of neuropathogenic EHV-1 infections. Based on the relatively fast course and fatal nature of EHV-1 infection in donkeys, it is worth studying the pathogenesis of EHM in donkeys both *in vivo* (*ex vivo*) and *in vitro*.

Together with the well-known association of genotype and differences in clinical signs, the season of the year, age, and gender have been reported as risk factors associated with EHV-1 infection (Goehring et al., 2006; Lunn et al., 2009). In our study, we recorded outbreaks of EHM during all seasons of the year, but a higher incidence of EHM was observed from April till mid-June. Strong seasonal clustering of EHM outbreaks has been described in The Netherlands (Goehring et al., 2006), where most outbreaks occurred between mid-November and mid-May. This seasonal clustering of EHM outbreaks was mainly associated with close confinement of equids (Goehring et al., 2006; Lunn et al., 2009). However, in Ethiopia, this temporal clustering of EHM outbreaks might be mainly due to stress associated with insufficient feed availability during these months of the year. Further, we observed EHM-affected equids mainly over 3 years of age and equids within the age ranged from 7-10 years were mostly affected. It has been reported in previous studies that adult horses were at a greater risk for developing EHM than young horses (Goehring et al., 2006; Barbic et al., 2012; Pusterla and Hussey, 2014). Although we clearly demonstrated that clinical EHM was largely restricted to equids over three years of age, the association of clinical manifestation of EHM and immunity remains to be determined. Mumford et al., (1987) isolated EHV-1 from the

nasopharynx of apparently healthy foals at the age of 7 - 9 days with high titers of maternal colostral antibody, during an abortion storm. This indicates that transfer of maternal virus neutralizing antibodies could not prevent infection, at least of the upper respiratory tract, but could provide clinical protection (Kydd et al., 2006).

The molecular epidemiological studies of EHV-1 have demonstrated that EHM is significantly associated with a SNP resulting in an amino acid variation of the EHV-1 DNA polymerase gene (ORF30) (Nugent et al., 2006; Goodman et al., 2007; Van de Walle et al., 2009). EHV-1 encoding aspartic acid (D_{752}) at amino acid position 752 has significantly a higher risk of causing neurological disease than those with the asparagine (N_{752}) (Nugent et al., 2006). In this thesis, we reported that 98.9% (90 out of 91) of equids with clinical signs of EHM were infected with ORF30 D_{752} variants, which indicated that the neuropathogenic variants were the predominant EHV-1 strains circulating in Ethiopian equids. It has been reported that more than 86% of EHM outbreaks are associated with strains encoding D_{752} (Nugent et al., 2006), whereas N_{752} variants have been responsible for approximately 95-98% of abortion outbreaks and between 15-25% of neurological outbreaks in the US, the UK, and other countries (Lunn et al., 2009). We also performed a phylogenetic analysis of ORF68 gene of EHV-1 isolates from Ethiopian equids. ORF68 has been described as a marker for strain variability that has shown some geographic restriction (Nugent et al., 2006) and helps to trace the source of the EHV-1 disease outbreaks (Barbic et al., 2012). In this study, we found that all the Ethiopian EHV-1 isolates belong to geographical group four. This group was mostly represented by the American and European EHV-1 isolates. In Ethiopia, there was no importation of equids from other countries and thus, it is very difficult to trace the origin of EHM outbreaks, leading the current problems.

Prevention of EHV-1 is difficult because many equids are latently infected, allowing the virus to circulate silently in equine populations, and the currently available modified live and inactivated vaccines are not reliably protective against the severe manifestations of the disease, including the fatal myeloencephalopathy. However, vaccination significantly reduces the severity of clinical outcome and viral nasal shedding. The study in the Netherlands and Belgium showed that more severe outbreaks of EHV-1 occurred in unvaccinated equine populations compared to vaccinated (Goehring et al., 2006; Gryspeerdt et al., 2011). Moreover, to date, no

specific therapy is effective against EHV-1 infections (Garré et al., 2007). Thus, early institution of appropriate disease control measures such as isolation of clinically diseased equids, segregation and monitoring of exposed equids, and quarantine measures should be implemented to prevent and control the spread of the virus. At present, there is no EHV-1 vaccine available in Ethiopia, and it is not clear to what extent the currently available vaccines may give protection for Ethiopian equids. Thus, more work is needed.

Equine herpesviruses in Ethiopian equids with and without respiratory problems

Respiratory pathogens are important causes of disease in equine populations worldwide. Previous studies have documented that respiratory problems, particularly coughing and nasal discharge are one of the major health concerns for working equids in Ethiopia (Laing et al., 2016). Equine herpesviruses are important pathogens that are involved in respiratory problems of varying severity. However, information regarding their prevalence in equids with and without respiratory disease is not available in Ethiopia. Therefore, in **chapter 3.2** of this thesis, we described the prevalence of EHV-1, -2, -4, and -5 and the genetic characterization of EHV-2 and EHV-5 from equids with and without respiratory disease. In chapter 3.1, we already described the EHV-1-associated myeloencephalopathy outbreaks in Ethiopian equids. In this study, we also provide the molecular evidence of EHV-1, EHV-2, EHV-4, and EHV-5 infection in Ethiopian donkeys and horses with and without respiratory problems. We detected EHV-5 at the highest prevalence (23.1%), followed by EHV-2 (20.0%), EHV-4 (8.1%), and EHV-1 (7.5%) from 160 equids showing clinical signs of respiratory disease. Of the total of 111 clinically healthy equids, we detected EHV-2 and EHV-5 from 8 (7.2%) and 18 (16.2%) horses, respectively, while EHV-1 and EHV-4 were never detected. It has been reported in previous studies that EHV-2 and EHV-5 were detected from immunocompetent equids without signs of respiratory syndromes (Bell et al., 2006; Wang et al., 2007; Torfason et al., 2008). Thus, we supported the notion that the presence of EHV-2 and/or EHV-5 in equids is not sufficient to establish disease causality (Brault and MacLachlan, 2011), however, they may compromise host immunity and increase the susceptibility of the host to opportunistic infections (Nordengrahn et al., 1996; Dunowska et al., 2002). Furthermore, we recorded concurrent infections with EHV-2 and EHV-5 from 9 (5.2%) clinically respiratory diseased horses. In previous studies, simultaneous infection with EHV-2 and EHV-5 have been reported

(Nordengrahn et al., 2002; Back et al., 2009; Ataseven et al., 2010). We observed dual infections from horses only exhibiting clinical signs of respiratory disease, therefore, it is important to evaluate the synergistic pathogenic effect of these viruses on the respiratory tract.

In this thesis, it was shown that both species of equids with respiratory clinical signs were infected with EHV-1, with a significantly higher prevalence in donkeys compared to horses. In chapter 3.1 of this thesis, a more severe clinical outcome and a larger proportion of donkeys infected with EHV-1 were reported, which indicated that the Ethiopian donkeys might be highly susceptible to EHV-1 infection. In contrast, we recorded a significantly higher prevalence of EHV-2 and EHV-5 in horses than in donkeys. It has been shown in previous studies that EHV-2 and EHV-5 have predominantly been found in horses (Franchini et al., 1997; Craig et al., 2005; Goehring, 2015). So far, information regarding infection of donkeys with either EHV-2 or EHV-5 has not been reported. However, infection of the Przewalski's wild horse and the mountain zebras with EHV-2 and EHV-5 were documented (Borchers et al., 1999). Thus, detection of EHV-2 and EHV-5 in donkeys is the first report, which provides an important contribution to a better understanding of the epidemiology of the disease. However, the varying level of susceptibility of donkeys and horses to EHV infections need to be further investigated.

Although there has been an unequivocal evidence that the alphaherpesviruses EHV-1 and EHV-4 are the major causes of respiratory disease in equids globally, the evidence that the gammaherpesviruses EHV-2 and EHV-5 as etiological agents of respiratory disease has not yet been defined. To this end, we compared the association of either EHV-2 or EHV-5 detection with the presence of respiratory clinical signs between equids displaying signs of respiratory disease and clinically healthy equids. In this study, we did not observe a significant difference in prevalence between EHV-5-infected equids in the respiratory diseased group and those not displaying signs of respiratory disease. Based on the presence of a high prevalence of EHV-5 in clinically normal equids coupled with the absence of a significant variation between the respiratory diseased and clinically healthy equids, we strongly suggested that EHV-5 was unlikely to be involved in respiratory disease in equids. In contrast, we observed a significantly higher proportion of EHV-2-infected equids in the respiratory disease group compared to those without disease and the EHV-2-positive equids were three-times more likely to display clinical signs of respiratory disease than EHV-2-negative equids. This strong association and low level

of detection from clinically healthy equids proposed that EHV-2 could be involved as an etiological agent either to induce or predispose equids to respiratory diseases. However, further studies are needed to better understand the clinical outcome of EHV-2 and EHV-5 infections.

In this thesis, we revealed a remarkable genetic diversity of the Ethiopian EHV-2 and EHV-5 strains based on the phylogenetic analysis of the gB gene with the nucleotide sequence homology among each other and with other countries isolates acquired from GenBank. We found that a nucleotide sequence homology of Ethiopian EHV-2 strains among each other and with foreign EHV-2 strains ranged from 94.0 to 99.4% and 92.9 to 99.1%, respectively. Similarly, EHV-5 strains showed 95.1 to 100% and 95.1 to 99.5% nucleotide sequence homology among each other and with isolates from other countries retrieved from GenBank, respectively. This high degree of genetic heterogeneity in equine gammaherpesviruses EHV-2 and EHV-5 have been reported in previous studies conducted elsewhere. Whether this genetic heterogeneity of the gammaherpesviruses has an association with the clinical outcome or not is not clear, thus, further investigation is needed.

Invasion characteristics of EHV-1 in the nasal and vaginal mucosae

Equids get the EHV-1 infection through inhalation of infectious aerosols or direct contact with infectious secretions. It is very well known that the respiratory mucosal epithelium is the primary site for EHV-1 replication. The virus breaches the basement membrane barriers by the use of infected mononuclear cells. Hereafter, the cell-associated viremia disseminates the virus to the target organs. Different strains of EHV-1 have a different pathological outcome, which is correlated with the variation in the ability to disseminate and establish infection in vascular endothelial cells of the target organs such as the endometrium, the central nervous system, or the eye (Patel et al., 1982; Hussey et al., 2013). It is essential to understand that different mucosal tissues are quite distinct in structural barriers, microenvironment, and the composition of available target cells. It was proposed that this unique characteristic of different mucosal tissues may dramatically influence the efficiency of EHV-1 replication. Several studies have been conducted on the replication kinetics and invasion mechanisms of EHV-1 in the equine respiratory mucosa. The replication efficiency in the vaginal mucosa was never studied before. The vaginal mucosa is the portal of entry for other alphaherpesviruses such as EHV-3 (Allen

and Umphenour, 2004), BoHV-1 (Bielanski et al., 2014; Wrathall et al., 2006), PRV (Romero et al., 2001) and CaHV-1 (Evermann et al., 2011). In previous studies, EHV-1 has been isolated from male genital organs (Tearle et al., 1996) and is shed with the semen and the sperm cells (Hebia-Fellah et al., 2009; Fritsche and Borchers, 2011; Walter et al., 2012). However, their transmission through the vaginal mucosa is not known, which could serve as an alternative portal of entry. Therefore, inspired by this knowledge, **in chapter 4** of this thesis, we used a mucosa tissue culture system, that mimics the natural conditions, to compare and evaluate the replication kinetics and invasion behavior of the neuropathogenic and abortigenic strains of EHV-1 in the nasal and vaginal mucosal tissue.

In this chapter, we demonstrated that EHV-l replicated and spread in a plaquewise manner in the epithelium and increases its size over time. We observed that EHV-1-infected mononuclear immune cells in the epithelium crossed the basement membrane and invaded the lamina propria of the respiratory and the vaginal mucosae. However, we noticed significantly different replication kinetics of EHV-1 in the nasal and vaginal mucosae, where both strains of EHV-1 replicated better in the nasal mucosa compared to the vaginal mucosa as evaluated by the number of plaques counted, the size of the plaques, and the number of infected individual mononuclear immune cells. This suggests that the nasal mucosa is the primary tissue of preference for EHV-1 replication and entry into its host.

Despite the fact that EHV-1 has a main tropism for the respiratory mucosa, the vaginal mucosa was able to support replication. In the vaginal mucosa, we observed a different magnitude of replication between the neuropathogenic and abortigenic strain. We clearly demonstrated that the neuropathogenic strain replicated less efficiently than the abortigenic strain and at 72h pi, we observed a lot of non-infected basal cells between the cluster of infected epithelial cells and the basement membrane for the neuropathogenic strain, but not for the abortigenic strain. The observed difference in replication efficiency between the two EHV-1 strains is associated with certain viral genetic factors. Goodman et al. (2007) reported that EHV-1 virulence and tissue tropism in the natural host are linked with the function of the DNA polymerase. Indeed, a single point mutation in this enzyme has been claimed to be responsible for the neurotropism. It is interesting to determine if this point mutation in this enzyme is also determining the level of EHV-1 replication in the vaginal mucosa.

In previous studies, the venereal shedding of EHV-1 from naturally infected stallions for three weeks was reported (Hebia-Fellah et al., 2009; Fritsche and Borchers, 2011; Walter et al., 2012). Based on this information, a possible venereal transmission of EHV-1 may be proposed. Hitherto, the transmission of EHV-1 through the vaginal mucosa was largely unknown. With our *ex vivo* explant models, we provided an important new insight for the possible venereal transmission of the virus. Studies with other herpesviruses showed that the viral dose is an important factor determining for transmission through the vaginal mucosa. It was demonstrated that venereal transmission of BoHV-1 via artificial insemination can occur with a dose as low as 32 $TCID_{50}$ in semen (Van Oirschot, 1995). Because the titer of EHV-1 shed with the semen has not been fully addressed, it is very important to determine this amount and evaluate its transmission either via artificial insemination or under natural conditions.

The monocytic cells represent an important cellular target for EHV-1 during primary infection. We demonstrated a variable number of EHV-1-infected monocytic cells in the nasal and vaginal mucosal tissues. Although the vaginal mucosa has multiple layered epithelial cells with many resident immune cells, we observed a two-to-five-fold lower percentage of EHV-1-infected monocytic cells in the lamina propria of the vaginal mucosa compared to the nasal mucosa. Based on this data, we proposed the hypothesis that the vaginal microenvironment may provide an important role to reduce the number of cells hijacked by the virus. However, this needs to be further investigated. Monocytic lineage cells, which express surface marker CD172a, were the predominant cell type infected with EHV-1, followed by $CD3^+$ T-lymphocytes independent of the strain and tissue. These monocytic cells serve as a "Trojan horse" to facilitate the dissemination of EHV-1 to target organs (Gryspeerdt et al., 2010; Vandekerckhove et al., 2010; Laval et al., 2015).

Invasion characteristics of EHV-3 in the nasal and vaginal mucosae

The alphaherpesvirus EHV-3 is the causal agent of equine coital exanthema, an acute venereal mucocutaneous disease of equids. EHV-3 is highly host specific and it replicates only in cell lines derived from equids (Allen and Umphenour, 2004; Barrandeguy et al., 2012). Although EHV-3 can establish genital and respiratory diseases, the underlying pathogenesis remains poorly understood. Therefore, in **chapter 4** of this study, we used the nasal and vaginal mucosal

explant model to elucidate the replication kinetics and early event of invasion behavior of EHV-3. Similar to EHV-1, we found a plaquewise manner replication characteristics of EHV-3. We observed the plaques in the epithelium starting from 24h pi and their sizes significantly increased over time. However, unlike EHV-1, we noticed restricted EHV-3 replication in both nasal and vaginal epithelial mucosae, where the virus neither breached the basement membrane to invade the lamina propria nor to infect individual mononuclear immune cells in all time points pi. This localized replication behavior might limit the EHV-3 dissemination via systemic blood circulation. Whether host factors or viral factors inhibit the virus to infect individual immune cells and to invade the underlying tissue is not clear and further investigation is recommended. Although both the nasal and vaginal mucosae can be infected with EHV-3, we observed a significantly strong replication in the vaginal mucosa than the nasal mucosa. This replication advantage of EHV-3 in the vaginal mucosa is associated with the clinical picture, as it causes vaginal lesions under natural conditions.

General conclusions and future directions

EHM is the most prevalent and devastating clinical outcome of the EHV-1-associated diseases in the Ethiopian equids. Donkeys and mules, besides horses, can be clinically affected by the neurological form of EHV-1. The neuropathogenic variant of EHV-1 is mainly responsible for the EHM outbreaks and all the EHV-1 isolates are allocated to geographical group 4. Because of the relatively fast course and fatal nature the EHV-1 infection in Ethiopian donkeys, future research should focus on elucidating the pathogenesis of EHM in donkeys using Ethiopian EHV-1 strains. In addition, the efficiency of EHV-1 vaccine has never been evaluated in donkeys and mules. Thus, it is important to evaluate the extent of protection using the currently available inactivated and live attenuated EHV-1 vaccines.

Respiratory problems associated with EHV infections are a major concern for Ethiopian equids. Both equine alphaherpesviruses (EHV-1 and EHV-4) and gammaherpesviruses (EHV-2 and EHV-5) are common respiratory tract pathogens in Ethiopian donkeys and horses. EHV-1 and EHV-4 are detected only from equids with respiratory diseases, whereas EHV-2 and EHV-5 are detected from both clinically ill and clinically healthy equids. In addition to EHV-1 and

EHV-4, EHV-2 is likely to be an important contributor either to induce or predispose equids to respiratory diseases.

The vaginal mucosa is susceptible to EHV-1 infection, with the abortigenic strain replicating more efficiently than the neuropathogenic strain. EHV-1 can infect the mononuclear cells residing in mucosa and hijack these cells to invade the underlying connective tissue. The $CD172a^+$ myeloid cells and $CD3^+$ T-lymphocytes are the predominant cell types infected by EHV-1. Because of the vaginal mucosa and residing immune cells are infected with EHV-1, it is important to further evaluate its venereal transmission *in vivo*.

EHV-3 replication is restricted to the epithelium of the nasal and vaginal mucosae and the virus neither breaches the basement membrane nor infect individual immune cells. EHV-3 replicates better in the genital mucosa than in the respiratory mucosa.

References

Allen, G., Umphenour, N., 2004. Equine Coital Exanthema, in: Tustin, J.C. and R. (Ed.), Infectious Diseases of Livestock. Oxford Press, Cape Town, South Africa, pp. 860–867.

Allen, G.P., Kydd, J.H., Slater, J.D., Smith, K.C., 2004. Equid herpesvirus 1 and equid herpesvirus 4 infections., in: JAW Coetzer and RC Tustin (Ed.), Infectious Diseases of Livestock. Oxford Press, Cape Town, South Africa, pp. 829–859.

Ataseven, V.S., Bilge-Dagalp, S., Oguzoglu, T.C., Karapinar, Z., Guzel, M., Tan, M.T., 2010. Detection and sequence analysis of equine gammaherpesviruses from horses with respiratory tract disease in Turkey. Transbound. Emerg. Dis. 57, 271–276.

Back, H., Ullman, K., Treiberg, L., Riihimäki, M., Penell, J., Ståhl, K., Valarcher, J., Pringle, J., 2009. Viral load of equine herpesviruses 2 and 5 in nasal swabs of actively racing Standardbred trotters: Temporal relationship of shedding to clinical findings and poor performance. Vet. Microbiol. 179, 142–148.

Barbic, L., Lojkic, I., Stevanovic, V., Bedekovic, T., Staresina, V., Lemo, N., Lojkic, M., Madic, J., 2012. Two outbreaks of neuropathogenic equine herpesvirus type 1 with breed-dependent clinical signs. Vet. Rec. 170, 227–227.

Barrandeguy, M., Vissani, A., Olguin, C., Barbara, G., Valenzuela, H., Becerra, L., Tordoya, M., Miño, S., Thiry, E., 2012. Experimental infection with equid herpesvirus 3 in seronegative and seropositive mares. Vet. Microbiol. 160, 319–326.

Bell, S.A., Balasuriya, U.B., Nordhausen, R.W., MacLachlan, N.J., 2006. Isolation of equine herpesvirus-5 from blood mononuclear cells of a gelding. J Vet Diagn Invest 18, 472–475.

Bielanski, A., Algire, J., Lalonde, A., Garceac, A., 2014. Risk of Transmission of Bovine Herpesvirus-1 (BHV-1) by Infected Semen to Embryo Recipients and Offspring. Reprod. Domest. Anim. 49, 197–201.

Borchers, K., Frölich, K., Ludwig, H., 1999. Detection of equine herpesvirus types 2 and 5 (EHV-2 and EHV-5) in Przewalski's wild horses. Arch. Virol. 144, 771–780.

Brault, S.A., MacLachlan, N.J., 2011. Equid gammaherpesviruses: Persistent bystanders or true pathogens? Vet. J. 187, 14–15.

Craig, M.I., Barrandeguy, M.E., Fernández, F.M., 2005. Equine herpesvirus 2 (EHV-2) infection in thoroughbred horses in Argentina. BMC Vet. Res. 1, 9.

Dunowska, M., Wilks, C.R., Studdert, M.J., Meers, J., 2002. Equine respiratory viruses in foals in New Zealand. N. Z. Vet. J. 50, 140–147.

Evermann, J.F., Ledbetter, E.C., Maes, R.K., 2011. Canine Reproductive, Respiratory, and Ocular Diseases due to Canine Herpesvirus. Vet. Clin. North Am. - Small Anim. Pract. 41, 1097–1120.

Franchini, M., Akens, M., Bracher, V., von Fellenberg, R., 1997. Characterisation of gamma herpesviruses in the horse by PCR. Virology 238, 8–13.

Friday, P. a, Scarratt, W.K., Elvinger, F., Timoney, P.J., Bonda, A., 2000. Ataxia and paresis with equine herpesvirus type 1 infection in a herd of riding school horses. J. Vet. Intern. Med. 14, 197–201.

Fritsche, A.K., Borchers, K., 2011. Detection of neuropathogenic strains of Equid Herpesvirus 1 (EHV-1) associated with abortions in Germany. Vet. Microbiol. 147, 176–180.

Garré, B., van der Meulen, K., Nugent, J., Neyts, J., Croubels, S., De Backer, P., Nauwynck, H., 2007. In vitro susceptibility of six isolates of equine herpesvirus 1 to acyclovir, ganciclovir, cidofovir, adefovir, PMEDAP and foscarnet. Vet. Microbiol. 122, 43–51.

Goehring, L.S., 2015a. Equid Herpesvirus–Associated Myeloencephalopathy, Seventh Ed. ed, Robinson's Current Therapy in Equine Medicine. Elsevier Inc.

Goehring, L.S., 2015b. γ-Herpesviruses in Horses and Donkeys, in: Robinson's Current

Therapy in Equine Medicine. Elsevier Inc., pp. 155–157.

Goehring, L.S., van Winden, S.C., van Maanen, C., Sloet van Oldruitenborgh-Oosterbaan, M.M., 2006. Equine herpesvirus type 1-associated myeloencephalopathy in The Netherlands: a four-year retrospective study (1999-2003). J. Vet. Intern. Med. 20, 601–607.

Goodman, L.B., Loregian, A., Perkins, G.A., Nugent, J., Buckles, E.L., Mercorelli, B., Kydd, J.H., Palù, G., Smith, K.C., Osterrieder, N., Davis-Poynter, N., 2007. A point mutation in a herpesvirus polymerase determines neuropathogenicity. PLoS Pathog. 3, 1583–1592.

Gryspeerdt, A., Vandekerckhove, A., Doorsselaere, J. Van, Nauwynck, H.J., 2011. Description of an unusually large outbreak of nervous system disorders caused by equine herpesvirus 1 (EHV1) in 2009 in Belgium. Vlaams Diergeneeskd. Tijdschr. 80, 147–153.

Gryspeerdt, A.C., Vandekerckhove, A.P., Garré, B., Barbé, F., Van de Walle, G.R., Nauwynck, H.J., 2010. Differences in replication kinetics and cell tropism between neurovirulent and non-neurovirulent EHV1 strains during the acute phase of infection in horses. Vet. Microbiol. 142, 242–253.

Hebia-Fellah, I., Léauté, A., Fiéni, F., Zientara, S., Imbert-Marcille, B.M., Besse, B., Fortier, G., Pronost, S., Miszczak, F., Ferry, B., Thorin, C., Pellerin, J.L., Bruyas, J.F., 2009. Evaluation of the presence of equine viral herpesvirus 1 (EHV-1) and equine viral herpesvirus 4 (EHV-4) DNA in stallion semen using polymerase chain reaction (PCR). Theriogenology 71, 1381–1389.

Henninger, R.W., Reed, S.M., Saville, W.J., Allen, G.P., Hass, G.F., Kohn, C.W., Sofaly, C., 2007. Outbreak of neurologic disease caused by equine herpesvirus-1 at a university equestrian center. J. Vet. Intern. Med. 21, 157–165.

Hussey, G.S., Goehring, L.S., Lunn, D.P., Hussey, S.B., Huang, T., Osterrieder, N., Powell, C., Hand, J., Holz, C., Slater, J., 2013. Experimental infection with equine herpesvirus type 1 (EHV-1) induces chorioretinal lesions. Vet. Res. 44, 1–15.

Kydd, J.H., Townsend, H.G.G., Hannant, D., 2006. The equine immune response to equine herpesvirus-1: The virus and its vaccines. Vet. Immunol. Immunopathol. 111, 15–30.

Laing, G., Christley, R., Stringer, A., Radford, A., Aklilu, N., Newton, R., Pinchbeck, G., 2016. Unraveling the causes of respiratory disease in the working equids of Ethiopia: A cross sectional survey. J. Equine Vet. Sci. 39, S98–S107.

Laval, K., Favoreel, H.W., Poelaert, K.C., Van Cleemput, J., Nauwynck, H.J., 2015. Equine

Herpesvirus Type 1 Enhances Viral Replication in CD172a+ Monocytic Cells upon Adhesion to Endothelial Cells. J. Virol. 89, 10912–10923.

Lunn, D.P., Horohov, D.W., Osterrieder, K., Pusterla, N., 2009. EHV-1 Consensus Statement. J Vet Intern Med 23, 450–461.

Mumford, J.A., Rossdale, P.D., Jessett, D.M., Gann, S.J., Ousey, J., Cook, R.F., 1987. Serological and virological investigations of an equid herpesvirus 1 (EHV-1) abortion storm on a stud farm in 1985. J Reprod Fertil Suppl. 35, 509–18.

Nordengrahn, A., Merza, M., Ros, C., Lindholmc, A., Palfl, V., Hannant, D., Belak, S., 2002. Prevalence of equine herpesvirus types 2 and 5 in horse populations by using type-specific PCR assays. Vet. Res. 33, 251–259.

Nordengrahn, A., Rusvai, M., Merza, M., Ekstrom, J., Morein, B., Belak, S., 1996. Equine herpesvirus type 2 (EHV-2) as a predisposing factor for Rhodococcus equi pneumonia in foals: Prevention of the bifactorial disease with EHV-2 immunostimulating complexes. Vet. Microbiol. 51, 55–68.

Nugent, J., Birch-Machin, I., Smith, K.C., Mumford, J.A., Swann, Z., Newton, J.R., Bowden, R.J., Allen, G.P., Davis-Poynter, N., 2006. Analysis of equid herpesvirus 1 strain variation reveals a point mutation of the DNA polymerase strongly associated with neuropathogenic versus nonneuropathogenic disease outbreaks. J. Virol. 80, 4047–4060.

Patel, J.R., Edington, N., Mumford, J.A., 1982. Variation in cellular tropism between isolates of Equine herpesvirus-1 in Foals. Arch. Virol. 74, 41–51.

Pusterla, N., Hussey, G.S., 2014. Equine herpesvirus 1 myeloencephalopathy. Vet. Clin. North Am. Equine Pract. 30, 489–506.

Pusterla, N., Mapes, S., Wademan, C., White, A., Estell, K., Swain, E., 2012. Investigation of the role of mules as silent shedders of EHV-1 during an outbreak of EHV-1 myeloencephalopathy in California. Vet. Rec. 170, 465–465.

Romero, C.H., Meade, P.N., Shultz, J.E., Chung, H.Y., Gibbs, E.P., Hahn, E.C., Lollis, G., 2001. Venereal transmission of pseudorabies viruses indigenous to feral swine. J. Wildl. Dis. 37, 289–296.

Tearle, J.P., Smith, K.C., Boyle, M.S., Binns, M.M., Livesay, G.J., Mumford, J.A., 1996. Replication of equid herpesvirus-1 (EHV-1) in the testes and epididymides of ponies and venereal shedding of infectious virus. J. Comp. Pathol. 115, 385–397.

Torfason, E.G., Thorsteinsdo´ttir, L., Thorsteinsdo´ttir, S.T., Svansson, V.S., 2008. Study of

equid herpesviruses 2 and 5 in Iceland with a type-specific polymerase chain reaction. Res. Vet. Sci. 85, 605–611.

USDA-APHIS, 2007. Equine herpesvirus myeloencephalopathy: a potential emerging disease. https://www.aphis.usda.gov/animal_health/emergingissues/downloads/ehv1final.pdf (accessed 7.7.16).

Van de Walle, G.R., Goupil, R., Wishon, C., Damiani, A., Perkins, G. a, Osterrieder, N., 2009. A single-nucleotide polymorphism in a herpesvirus DNA polymerase is sufficient to cause lethal neurological disease. J. Infect. Dis. 200, 20–25.

Van Maanen, C., 2002. Equine herpesvirus 1 and 4 infections: An update. Vet. Q. 24, 58–78.

Van Oirschot, J.T., 1995. Bovine herpesvirus 1 in semen of bulls and the risk of transmission: a brief review. Vet. Q. 17, 29–33.

Vandekerckhove, A.P., Glorieux, S., Gryspeerdt, A.C., Steukers, L., Duchateau, L., Osterrieder, N., Van De Walle, G.R., Nauwynck, H.J., 2010. Replication kinetics of neurovirulent versus non-neurovirulent equine herpesvirus type 1 strains in equine nasal mucosal explants. J. Gen. Virol. 91, 2019–2028.

Walter, J., Balzer, H.J., Seeh, C., Fey, K., Bleul, U., Osterrieder, N., 2012. Venereal Shedding of Equid Herpesvirus-1 (EHV-1) in Naturally Infected Stallions. J. Vet. Intern. Med. 26, 1500–1504.

Wang, L., Raidal, S.L., Pizzirani, A., Wilcox, G.E., 2007. Detection of respiratory herpesviruses in foals and adult horses determined by nested multiplex PCR. Vet. Microbiol. 121, 18–28.

Wrathall, A.E., Simmons, H.A., Van Soom, A., 2006. Evaluation of risks of viral transmission to recipients of bovine embryos arising from fertilisation with virus-infected semen. Theriogenology 65, 247–274.

CHAPTER 6

SUMMARY - SAMENVATTING

Summary

The equine alphaherpesviruses (EHV-1, EHV-3, and EHV-4) and gammaherpesviruses (EHV-2 and EHV-5) are ubiquitous pathogens in equine populations worldwide. They establish a lifelong latent infection, which ensures the survival of herpesviruses in equine populations. The currently available modified live and inactivated products of EHV-1 and EHV-4 vaccines are not reliably providing protection against infection. A better understanding of the epidemiology of EHV infections in natural outbreaks is particularly important to formulate an effective intervention strategy. However, many data gaps exist and more investigation needs to be done. It is very well known that the respiratory mucosal epithelium is the primary site of EHV-1 replication. However, EHV-1 infection in the vaginal mucosa is largely unknown. Moreover, information regarding the pathogenesis of EHV-3 in the nasal and vaginal mucosa is limited. Therefore, this thesis aimed to describe the epidemiology of EHVs in Ethiopian equids and to elucidate early events of EHV-1 and EHV-3 invasion characteristics by the use of respiratory and vaginal mucosal explant system.

In Chapter 1, an overview of the current knowledge of EHVs and the characteristics of the respiratory and genital mucosae were given. In this chapter, an introduction was given on the historical background, the taxonomy, the general herpesvirion structure, the replication cycle, the current understanding of the epidemiology, the pathogenesis and the EHV-associated clinical signs. We also provided the general characteristics of upper respiratory and genital tract mucosal tissues with residing immune cells.

In Chapter 2, the aims of this thesis were formulated.

In Chapter 3.1, the epidemiology and molecular characteristics of EHV-1 from clinically EHM-affected Ethiopian equids during outbreaks from May 2011 to December 2013 were described. We recorded a higher incidence of EHM outbreaks from April to mid-June. There were no visible differences in the clinical presentation of EHM among horses, mules, and donkeys. However, EHM in donkeys was more severe and regularly death occurred after a short period of neurological signs. EHM-affected equids mainly observed over 3 years of

age and equids within the age ranged from 7 - 10 years (n = 51; 56.0%) were mostly affected. Further, we also documented that females were more affected than males. The incidence of neuropathogenic (D_{752}) and non-neuropathogenic (N_{752}) variants of EHV-1 from EHM-affected Ethiopian equids was assessed by sequencing the DNA polymerase gene (ORF30) of the EHV-1 isolates. The results indicated that 98.9% (90 out of 91) equids were infected with an ORF30 D_{752} variant. Analysis of ORF68 as grouping marker for geographical differences showed that the Ethiopian EHV-1 isolates belonged to geographical group 4. Due to the fatal nature of EHV-1 in donkeys, it would be interesting to examine the pathogenesis of EHM in this species. At present, there is no vaccine available in Ethiopia, and therefore, outbreaks of EHV-1 should be controlled by proper management adaptations. In addition, it is important to test the efficacy of the commercial vaccines not only in horses but also in donkeys and mules.

In Chapter 3.2, we described the detection and genetic characterization of EHVs from equids with and without clinical respiratory disease. Virus-specific PCRs were used to detect EHV-1, -2, -4, and -5. From the total of 160 equids with respiratory disease, EHV-5 was detected at the highest prevalence (23.1%), followed by EHV-2 (20.0%), EHV-4 (8.1%), and EHV-1 (7.5%). Concurrent infections with EHV-2 and EHV-5 were recorded from 9 (5.2%) diseased horses. Of the total of 111 clinically healthy equids, EHV-1 and EHV-4 were never detected whereas EHV-2 and EHV-5 were found in 8 (7.2%) and 18 (16.2%) horses, respectively. A significantly higher proportion of EHV-2-infected equids was observed in the respiratory disease group (32/160, 20.0%; P = 0.005) compared to those without disease (8/111; 7.2%). EHV-2-positive equids were three times more likely to display clinical signs of respiratory disease than EHV-2-negative equids (OR 3.22, 95% CI: 1.42 to 7.28). For EHV-5, the observed difference was not significantly (P = 0.166) different. The phylogenetic analysis of the gB gene revealed that the Ethiopian EHV-2 and EHV-5 strains had a remarkable genetic diversity, with a nucleotide sequence identity among each other that ranged from 94.0 to 99.4% and 95.1 to 100%, respectively. Moreover, the nucleotide sequence identity of EHV-2 and EHV-5 with isolates from other countries acquired from GenBank ranged from 92.9 to 99.1% and 95.1 to 99.5%, respectively. Our results suggest that besides EHV-1 and EHV-4, EHV-2 is likely to be an important contributor either to induce or predispose equids to respiratory disease. However, more work

is needed to better understand the contribution of EHV-2 in the establishment of respiratory disease.

In Chapter 4, the replication kinetics and invasion characteristics of EHV-1 and EHV-3 in nasal and vaginal mucosae were compared using explants. The tissues were inoculated with EHV-1 03P37 (neuropathogenic), 97P70 (abortigenic), and EHV-3 04P57. The explants were collected at 0, 24, 48, and 72h pi, cryosections were made, fixed and stained for viral antigens by a double immunofluorescence labeling. The plaques were already observed at 24 h pi, their size increased over time and did not directly cross the basement membrane. However, EHV-1 infected the monocytic cells and hijacked these cells to invade the lamina propria. In contrast, EHV-3 replication was fully restricted to epithelial cells; the virus did not breach the basement membrane via a direct cell-to-cell spread nor used infected monocytic cells. EHV-1-induced plaques were larger in the nasal mucosa compared to the vaginal mucosa. The opposite was found for EHV-3-induced plaques. Both EHV-1 strains replicated with comparable kinetics in the nasal mucosa. However, the extent of replication of the abortigenic strain in vaginal mucosa was significantly higher than that of the neuropathogenic strain. Two-to-five-fold lower numbers of EHV-1-infected monocytic cells underneath the BM were found in the vaginal mucosa than in the nasal mucosa. Our study has shown that (i) EHV-1 has developed in evolution a predisposition for respiratory mucosa and EHV-3 for vaginal mucosa, (ii) abortigenic EHV-1 replicates better in vaginal mucosa than neuropathogenic EHV-1 and (iii) EHV-3 demonstrated a strict epithelial tropism whereas EHV-1 in addition hijacked monocytic cells to invade the lamina propria.

In **Chapter 5**, all data obtained in the present thesis were reviewed and discussed. A general conclusion on the research data generated in this thesis was formulated, in which the epidemiology of EHVs in Ethiopian equids and the new insights in the invasion behavior of EHV-1 and EHV-3 in the respiratory and vaginal mucosae were summarized.

Samenvatting

De equine alfaherpesvirussen (EHV-1, EHV-3 en EHV-4) en gammaherpesvirussen (EHV-2 en EHV-5) zijn alomtegenwoordig in paardenpopulaties wereldwijd. Ze veroorzaken een levenslange latente infectie waardoor het virus na de acute infectie aanwezig zal blijven in de gastheer. De beschikbare levende en geïnactiveerde EHV-1 producten en de EHV-4 vaccins leiden niet tot een betrouwbare bescherming tegen infectie. Het beter begrijpen van de epidemiologie van EHV-infecties bij natuurlijke uitbraken is vooral belangrijk om een effectieve interventiestrategie te formuleren. Er zijn echter nog veel hiaten in de kennis hieromtrent en meer onderzoek dringt zich op. Het is gekend dat het respiratoire epitheel de primaire vermeerderingsplaats van EHV-1 is. Of EHV-1 ook de vaginale mucosa kan infecteren, is niet geweten. Verder is er weinig informatie over de replicatie-eigenschappen van EHV-3 in nasale en vaginale mucosae bekend. Het doel van deze thesis is daarom de epidemiologie van EHVs in Ethiopische paardachtigen te beschrijven en de vroege pathogenese van EHV-1 en EHV-3 infecties te verhelderen door gebruik te maken van respiratoire en vaginale mucosale explantsystemen.

In Hoofdstuk 1 wordt een overzicht gegeven van de huidige kennis van de EHVs en de karakteristieken van de respiratoire en vaginale slijmvliezen (mucosae). In dit hoofdstuk wordt een inleiding gegeven over de historische achtergrond, de taxonomie, de algemene herpesvirionstructuur, de vermeerderingscyclus, de huidige kennis van de epidemiologie, de pathogenese en symptomen van een EHV-infectie. We overlopen ook de algemene kenmerken van de slijmvliezen van de bovenste luchtwegen en de voortplantingsorganen met residentiële immuuncellen.

In Hoofdstuk 2 worden de doelstellingen van de thesis uiteengezet.

In Hoofdstuk 3.1 wordt de epidemiologie beschreven, alsook de genetische karakterisatie van EHV-1 van klinisch EHM-geïnfecteerde Ethiopische paardachtigen tijdens uitbraken van mei 2011 tot december 2013. We namen een hogere incidentie van EHM uitbraken waar van april tot half juni. Er waren geen zichtbare verschillen in de klinische presentatie van EHM tussen paarden, muilezels en ezels. Echter, EHM in ezels was ernstiger en frequent

werd sterfte zonder veel voorafgaande tekenen van paralyse waargenomen. EHM werd voornamelijk bevonden bij paardachtigen ouder dan 3 jaar en de overgrote meerderheid van de gevallen trof paarden tussen 7 en 10 jaar oud. Verder hebben we ook gedocumenteerd dat merries vaker geïnfecteerd werden dan hengsten. De incidentie van neuropathogene (D752) en niet-neuropathogene (N752) varianten van EHV-1 van EHM-geïnfecteerde Ethiopische paardachtigen werd bepaald door sequentiebepaling van het DNA polymerase gen (ORF30) van de EHV-1 isolaten. De resultaten gaven aan dat 98,9% (90 van 91) van de paardachtigen werden geïnfecteerd met een D752 ORF30 variant. Analyse van ORF68 als groepsmerker voor geografische verschillen toonde aan dat de Ethiopische EHV-1 isolaten behoren tot de geografische groep 4. Door de fatale aard van EHV-1 in ezels zou het interessant zijn om de pathogenese van EHM te onderzoeken in deze diersoort. Op dit moment is er geen vaccin beschikbaar in Ethiopië waardoor EHV-1 uitbraken enkel kunnen bestreden worden door aangepaste managementmaatregelen. Bovendien is het belangrijk om de werkzaamheid van de commerciële vaccins niet alleen bij paarden, maar ook bij ezels en muildieren te testen.

In Hoofdstuk 3.2 beschrijven we de detectie en de genetische kenmerken van EHVs van paardachtigen met en zonder klinische respiratoire aandoeningen. Virus-specifieke PCRs werden gebruikt om EHV-1, -2, -4 en -5 te detecteren. Van het totaal van 160 paardachtigen met ademhalingsaandoeningen was de prevalentie het hoogst voor EHV-5 (23,1%), gevolgd door EHV-2 (20,0%), EHV-4 (8,1%) en EHV-1 (7,5%). Gelijktijdige infecties met EHV-2 en EHV-5 werden waargenomen bij 9 (5,2%) zieke paarden. Bij de in totaal 111 klinisch gezonde paardachtigen werden EHV-1 en EHV-4 nooit gedetecteerd terwijl EHV-2 en EHV-5 werden gevonden bij 8 (7,2%) en 18 (16,2%) paarden, respectievelijk. Een significant groter aandeel van EHV-2 geïnfecteerde paardachtigen werd waargenomen bij de groep met ademhalingsstoornissen (32/160, 20,0%; P = 0,005) in vergelijking met dieren zonder ziekte (8/111; 7,2%). EHV-2-positieve paardachtigen hadden drie keer meer kans om symptomen van ziekte van de luchtwegen te ontwikkelen dan EHV-2-negatieve paardachtigen (OR 3,22, 95% CI: 1,42-7,28). Voor EHV-5 was het waargenomen verschil niet significant (p = 0,166) verschillend. De fylogenetische analyse van het gB gen bracht aan het licht dat de Ethiopische EHV-2 en EHV-5 stammen een opmerkelijke genetische diversiteit vertonen, met een overeenstemming in nucleotide sequentie die varieert van

respectievelijk 94,0 tot 99,4% en van 95,1 tot 100%. Bovendien varieerde de nucleotidesequentie overeenstemming van EHV-2 en EHV-5 met isolaten uit andere landen, verkregen van GenBank, van respectievelijk 92,9 tot 99,1% en van 95,1 tot 99,5%. Onze resultaten suggereren dat naast EHV-1 en EHV-4, EHV-2 waarschijnlijk een belangrijke bijdrage levert aan het veroorzaken van ademhalingsziekte bij paardachtigen of paardachtigen vatbaarder maakt. Er is echter meer werk nodig om beter inzicht te krijgen in de bijdrage van EHV-2 in het veroorzaken van respiratoire aandoeningen.

In Hoofdstuk 4 worden de kinetieken de invasiekenmerken van EHV-1 en EHV-3 in nasale en vaginale mucosae vergeleken met behulp van explanten. De weefsels werden geïnoculeerd met EHV-1 stammen 03P37 (neuropathogeen), 97P70 (abortigeen) en EHV-3 stam 04P57. De explanten werden verzameld op 0, 24, 48 en 72 uur na infectie, en cryosecties werden gemaakt, gefixeerd en gekleurd voor virale antigenen met dubbele immunofluorescentiemarkering. De plaques werden reeds waargenomen vanaf 24 uur na infectie, hun omvang nam toe met de tijd en braken niet direct door de basale membraan. Echter, EHV-1 infecteerde monocytaire cellen en gebruikte deze cellen om de lamina propria te kunnen binnendringen. Daartegenover bleef de EHV-3 replicatie beperkt tot de epitheelcellen; het virus bracht geen schade toe aan de basaalemembraan via een directe cel-cel spreiding noch gebruikte het virus monocytaire cellen om de basaalemembraan te penetreren. EHV-1-geïnduceerde plaques waren groter in het neusslijmvlies ten opzichte van die in het vaginale slijmvlies. Het tegenovergestelde werd gevonden voor EHV-3 geïnduceerde plaques. Beide EHV-1 stammen vermeerderden even sterk in het neusslijmvlies. De omvang van vermeerdering van de abortigene stam in de vaginale mucosa was significant groter dan de vermeerdering van de neuropathogene stam. Het aantal EHV-1-geïnfecteerde monocytaire cellen onder de basaal membraan was twee tot vijf maal lager in de vaginale mucosa dan in het neusslijmvlies. Ons onderzoek heeft aangetoond dat (i) EHV-1 in de evolutie een voorkeur voor de respiratoire mucosa ontwikkelde en EHV-3 voor de vaginale mucosa, (ii) de abortigene EHV-1 stam beter vermeerdert in het vaginale slijmvlies dan de neuropathogene EHV-1 stam en (iii) EHV-3 een strikte epitheliale voorkeur heft, terwijl EHV-1 ook gebruik maakt van monocytaire cellen om de lamina propria binnen te dringen.

In Hoofdstuk 5 worden alle verkregen gegevens in dit proefschrift beoordeeld en besproken. Algemene conclusie van de onderzoeksgegevens werd geformuleerd, waarin de epidemiologie van EHVs bij Ethiopische paardachtigen en de nieuwe inzichten in het invasieve gedrag van EHV-1 en EHV-3 in de luchtwegen en de vaginale slijmvliezen worden samengevat.

CURRICULUM VITAE

Haileleul Negussie Dubale was born in Ethiopia. In August 2002, he received a degree of Doctor of Veterinary Medicine (DVM) from the Faculty of Veterinary Medicine at the Addis Ababa University, Ethiopia. In October 2002, he started his professional career as a lecturer in the Department of Animal Health, Alage Technical and Vocational Education and Training College. In September 2007, he continued his master studies in the Faculty of Veterinary Medicine at the Addis Ababa University, Ethiopia. He did his master thesis with a project on "molecular epidemiology and vaccine matching study on foot and mouth disease virus isolated in Ethiopia". In June 2009, he obtained a Master's degree in Veterinary Epidemiology. Haileleul joined the Faculty of Veterinary Medicine at Addis Ababa University, Ethiopia in 2009 as an Assistant Professor of epidemiology and preventive medicine. In October 2012, he started a PhD program under the supervision of Professor Hans Nauwynck in the Laboratory of Virology, Faculty of Veterinary Medicine at Ghent University. During his PhD studies, he worked on equine herpesviruses. His PhD program was funded by "The Special Research Fund (BOF)" of the Ghent University. He is author and co-author of several publications in national and international peer-reviewed journals.

Selected publications in international peer-reviewed journals

Negussie H, Li Y, Tessema TS, Nauwynck HJ. Replication characteristics of equine herpesvirus 1 and equine herpesvirus 3: comparative analysis using ex vivo tissue cultures. Vet Res. 2016, 47:19. PubMed PMID: 26768993.

Negussie H, Gizaw D, Tessema TS, Nauwynck HJ. Equine Herpesvirus-1 myeloencephalopathy, an emerging threat of working equids in Ethiopia. Transbound Emerg Dis. 2015 May 26. PubMed PMID: 26010868.

Negussie H, Gizaw D, Tesfaw L, Oguma L, Sentsui H, Tessema TS, Nauwynck HJ: Detection of equine herpesvirus (EHV) -1, -2, -4, and -5 in Ethiopian equids with and without respiratory problems and genetic characterization EHV-2 and EHV-5 strains. Transbound Emerg Dis. (2016), DOI:10.1111/tbed.12601.

Zhao J, **Negussie H**, Laval K, Poelaert KC, Nauwynck HJ. Dual infections of equine herpesvirus 1 and equine arteritis virus in equine respiratory mucosa explants. Virus Res. 2016, 15; 220:104-11. PubMed PMID: 27117322.

Li Y, **Negussie H**, Qiu Y, Reddy VR, Mateusen B, Nauwynck HJ. Early events of canine herpesvirus 1 infections in canine respiratory and genital mucosae by the use of ex vivo models. Res Vet Sci. 2016, 105:205-8. PubMed PMID: 27033934.

Negussie H, Kyule MN, Yami M, Ayelet G, Jenberie S. Outbreak investigations and genetic characterization of foot-and-mouth disease virus in Ethiopia in 2008/2009. Trop Anim Health Prod. 2011, 43(1):235-43. PubMed PMID: 20717724.

Negussie H, Ayelet G, Jenberie S, Minda, Tesfaw L. Molecular epidemiology and vaccine matching study on foot-and-mouth disease virus circulating in Ethiopia. African Journal of Microbiology Research: 2013, 7(44):5101-5106.

Jenberie S, Lynch SE, Kebede F, Christley RM, Gelaye E, **Negussie H**, Asmare K, Ayelet G. Genetic characterization of infectious bursal disease virus isolates in Ethiopia. Acta Trop. 2014, 130:39-43. PubMed PMID: 24145155.

Legesse Y, Asfaw Y, Sahle M, Ayelet G, Jenberie S, **Negussie H**. First confirmation of foot and mouth disease virus serotype SAT-1 in cattle and small ruminants in Ethiopia in 2007/08. Trop Anim Health Prod. 2013, 45(5):1265-7. PubMed PMID: 23250672.

Ayelet G, Abate Y, Sisay T, **Negussie H**, Gelaye E, Jemberie S, Asmare K. Lumpy skin disease: preliminary vaccine efficacy assessment and overview on outbreak impact in dairy cattle at Debre Zeit, central Ethiopia. Antiviral Res. 2013, 98(2):261-5. PubMed PMID: 23428671.

Jenbreie S, Ayelet G, Gelaye E, Kebede F, Lynch SE, **Negussie H**. Infectious bursal disease: seroprevalence and associated risk factors in major poultry rearing areas of Ethiopia. Trop Anim Health Prod. 2013, 45(1):75-9. PubMed PMID: 22639033.

Ayelet G, Gelaye E, **Negussie H**, Asmare K. Study on the epidemiology of foot and mouth disease in Ethiopia. Rev Sci Tech. 2012, 31(3):789-98. PubMed PMID: 23520733.

Kebede H, Lemma A, **Negussie H.** Ultrasonographic studies on ovarian dynamics and associated estrus manifestations of jennies under controlled management, Ethiopia. Trop Anim Health Prod. 2012, 44(8):1965-70. PubMed PMID: 22581394.

Jenberie S, Awol N, Ayelet G, Gelaye E, **Negussie H**, Abie G. Gross and histopathological studies on pulmonary lesions of camel (Camelus dromedarius) slaughtered at Addis Ababa abattoir, Ethiopia. Trop Anim Health Prod. 2012, 44(4):849-54. PubMed PMID: 21909867.

Berhanu W, **Negussie H,** Alemu S, Mazengia H. Assessment on major factors that cause skin rejection at Modjo export tannery, Ethiopia. Trop Anim Health Prod. 2011, 43(5):989-93. PubMed PMID: 21327713.

Yesuf M, Alemu S, Temesgen W, Mazengia H, **Negussie H.** Seroprevalence of ovine brucellosis in south Wollo, northeastern Ethiopia. East Afr J Public Health. 2011, 8(1):58-60. PubMed PMID: 22066286. 5.

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